AI-2-LIKE ACTIVITY MEDIATED *E. coli* O157:H7 SURVIVAL AND VIRULENCE GENE EXPRESSION IN THE PRESENCE OF GROUND BEEF EXTRACTS

A Thesis

by

KAMLESHKUMAR ARVINDKUMAR SONI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2005

Major Subject: Food Science and Technology

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May 2005

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ABSTRACT

AI-2-like Activity Mediated *E. coli* O157:H7 Survival and Virulence Gene Expression in the Presence of Ground Beef Extracts. (May 2005)

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Cell-to-cell communication, termed quorum sensing, mediated by AI-2 like activity, has been reported to regulate the expression of a variety of genes in *E. coli* O157:H7. A previous study in our laboratory has shown that foods can contain compounds that can interfere with AI-2 signaling. The underlying hypothesis of our studies is that the autoinducer molecules such as AI-2 are involved in the virulence and survival of enteric bacterial pathogens on food and food ingredients. The influence of AI-2 like activity on the survival and expression of virulence genes (*hha* and *yadK*) in *E.coli* O157:H7 was studied when the organism was stored in different types of ground beef extracts such as: cooked, uncooked, and autoclaved. The survival was observed at refrigeration temperature, while change in gene expression was studied using real-time PCR. Higher survival was observed in the cell exposed to cell free supernatant (CFS) containing AI-2 like molecules, compared to the one which was exposed to heat degraded AI-2 like molecules. The survival of cells was higher when exposed to cooked ground beef extracts compared to uncooked and autoclaved ground beef extracts. Similarly, higher gene expressions of both *hha* and *yadK* genes were observed in cells that were exposed to cooked beef extract samples as compared to samples that were

uncooked or autoclaved. About a 2 fold higher gene expression for both *hha* and *yadK* gene was observed when cells were subjected to cooked ground beef extracts in the presence of AI-2 like molecules compared to the ones exposed to uncooked ground beef extracts in the presence of AI-2 like molecules. Likewise, 3-fold higher gene expression was observed for cells exposed to cooked ground beef extracts compare to autoclaved ground beef extracts in the presence of AI-2 like molecules. The results suggest that the survival and virulence of enteric bacterial pathogens such as *E.coli* O157:H7 can be influenced by the interaction of food components and autoinducers such as AI-2, that are involved in bacterial cell communications.

DEDICATION

To my parents whose patience and support gave me the ability.

To my uncle, aunty and cousins whose support made my dream come true.

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INTRODUCTION

RATIONALE

Bacterial cell-to-cell communication, termed quorum sensing and mediated by autoinducers, has been reported to regulate a variety of microbial processes such as virulence, antibiotic resistance, sporulation, and motility (Ren et al., 2004; Sperandio et al., 2003, Smith et al., 2004). Optimal levels of these cell signaling (autoinducer) molecules and interaction of these molecules with regulatory proteins are key steps that are involved in the coordination of gene expression. Over the last decade, a significant amount of work has been conducted on quorum sensing at the molecular level (Lu et al., 2004; Smith et al., 2004). However, the knowledge gained so far has had limited applicability to the food industry, because of limited knowledge about the role and interaction of autoinducer molecules with foods and food ingredients (Lu et al., 2004; Smith et al., 2004). It is important to understand the effect of autoinducer molecules on the behavior of bacterial processes such as virulence, gene expression, survival, toxin production, and sporulation when present in foods. It is imperative to understand these effects, because foods may contain compounds that can repress or enhance cell-cell communication. In addition, the behavior of bacterial populations in different foods can be dependant on the levels of autoinducer

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molecules in foods. Moreover, since foods are complex ecosystems, which can undergo large physico-chemical changes during various processing steps, the fate and behavior of microorganisms can be completely different at different processing conditions.

Escherichia coli, an intestinal commensal organism of bovine animals, is responsible for a number of food borne outbreaks (Griffin et al.1991). Ground beef serves as a major vehicle for the transfer of *E. coli* O157:H7 from bovine animals to humans. Even though there have been continuous efforts to control the incidence of *E. coli* O157:H7 in foods, there was not much difference in the annual numbers of cases observed between 1996 and 2002 (MMWR 2003). Fewer food borne cases were related to *E. coli* O157:H7 than other pathogenic organisms, such as *Salmonella*, *Campylobacter* and *Listeria* (MMWR, 2003, MMWR, 2004; Mead et al; 1999)**.** Even though the occurrence of *E. coli* O157:H7 is less compared to these other organisms, it is a serious food safety risk, because of its low infectious dose. Furthermore, the ability of *E. coli* O157:H7 to survive in a low pH environment and in refrigerated products makes it of serious concern (Mamani et al., 2003; Bachrouri et al., 2002). Though intervention technologies such as better produce production practices, irradiation, and pasteurization have been successful in controlling the food borne illness associated with *E. coli* O157:H7, there is much room for improvement.

Previous studies in our laboratory have shown that foods can contain inhibitory compounds that can interfere with AI-2 signaling (Lu et al., 2004). We are trying to develop a radically new approach to control the survival and virulence gene expression of *E. coli* O157:H7 by interrupting bacterial cell-cell communication signals using these

inhibitory compounds. This new approach of interpreting basic communication signals between the bacterial cells is based on AI-2 molecules and AI-2-like activity. The advantage of this new approach is that in future we may not have to rely on the introduction of chemicals in food processing as an intervention tool. In addition, the inherent inhibitory compounds that are naturally present in certain meat components can potentially have value as food additives. Since these inhibitory compounds are considered as Generally Recognized as Safe (GRAS), they can play a major role in the development of the next generation intervention of technologies. Furthermore, as these compounds are GRAS they will not require an extensive procedure for approval as food additives. In this work, I have attempted to understand how AI-2 autoinducer molecules behave and regulate the bacterial processes such as, survival and gene expression of two virulence related genes in *E. coli* O157:H7, in the presence of naturally occurring inhibitory compounds in ground beef.

The underlying hypothesis is that the naturally occurring AI-2-like activity inhibitory compounds in ground beef extracts can limit the survival and expression of the selected virulence genes.

OBJECTIVES

The primary objectives of this study are:

1. To understand the effect of AI-2-like molecules on the survival of *E. coli* O157:H7 in ground beef extract that were subjected to uncooked, cooked, and autoclaved conditions.

2. To understand the effect of AI-2-like activity on the expression of two virulence-related genes (*hha* and *yadK*) in *E. coli* O157:H7 when exposed to cooked, uncooked and autoclaved ground beef extracts.

E. coli O157:H7 has ability to survive under different food environmental conditions such as low pH (orange juice), low water activity (chocolate), and refrigeration (ground beef, milk). The survival study of *E. coli* O157:H7 in ground beef is important because the organism is frequently associated with ground beef associated outbreaks and it has the ability to survive for a long time at refrigeration temperature. The *hha* gene is involved in the production of the hemolysis modulation protein, while the *yadK* gene is involved in the production of putative fimbrial-like protein. The *hha* gene is involved in modulating the gene involved in hemolysis of cells while *yadK* gene is related to motility and secretion (Welch et al., 2002; IFGE, 2004).

LITERATURE REVIEW

BACTERIAL QUORUM SENSING

Quorum sensing regulates a variety of bacterial processes by inter-and intra signaling molecules termed autoinducers (Bassler et al., 2002; Sperandio et al. 2003). These autoinducer molecules are believed to control various bacterial processes in response to environmental stimuli. Numerous reports have shown how these molecules can regulate bacterial processes in both Gram-positive and Gram-negative bacteria (Miller et al.2001; Delisa et al., 2001; Jesper at al., 2004)**.** Bacteria secrete or diffuse the autoinducer molecules, which attach to the same or other cells and activate the membrane receptors (Miller et al., 2001). When these molecules attain a sufficient concentration in environment, they elicit the induction of trans membrane signals that leads to the control of global gene expression and ultimately to the control of various bacterial processes **(**Miller et al., 2001; Smith et al., 2004). As per present knowledge, bacterial quorum sensing molecules can be divided into four different categories: 1) N-acyl homoserine lactones (AHLs), also called AI-1 molecules and used by Gram-negative bacteria for intra-species communications (Miller et al., 2001); 2) a furanosyl borate diester, molecule termed AI-2 and is used by both inter-and intra- species communications (Chen et al., 2002); 3) AI-3 molecules of unknown structures, used in the bacterial- host communication (Sperandio et al., 2003); 4) Peptides, called autoinducer peptides (AIP) and short chain amino acids, used by Gram-positive bacteria (Miller et al. 2001, Smith et al., 2004, Chan et al.,2004). AI-1 molecules are solely

involved in the intra-species communication in Gram-negative bacteria, while AI-2 molecules are involved in inter-species communication and are used by both Gram-negative and Gram-positive bacteria (Smith et al., 2004). Recently, Miller et al. (2004) has shown that S. *Typhimurium* can recognize a chemically distinct form of the AI-2 molecules. LuxP is the binding protein for conventional AI-2 molecules, while LsrB is the binding protein for the distinct form (Miller et al. 2004). This distinct form of AI-2 molecules differs from the conventional form in two aspects: 1) The distinct form of AI-2 molecules does not contain boron in its ring structure, while the conventional AI-2 molecules do contain boron; 2) The LuxP protein contains a net positive charge that favors the binding of conventional AI-2 molecules, which has a negative charge in the boron moiety, while LsrB contains a net negative charge and does not bind to boron. Presently, many bacterial processes are known to be controlled by these quorum sensing molecules. In the future, it is highly likely that we may find many more autoinducer molecules. Until now, the majority of work done with AI-2 is based on using cell free supernatants, in which activity is detected by luminance response in the reporter strain of *V. harveyi*. Because of this reason, rather than using term AI-2 molecules or AI-3 molecules, I have purposely mentioned it as "AI-2-like activity" or "AI-2-like molecules".

The quorum sensing system in *E. coli* O157:H7 is well studied. Of the entire *E coli* genome, about 5-10% genes are controlled by AI-2-like molecules (Delisa et al., 2001; Sperandio et al., 2001). Furthermore, Ren et al. (2004) have shown that 79% (44 out of 56) of *E. coli* genes are repressed by furanone, and which are otherwise induced

by AI-2-like molecules. Even though significant amount of laboratory work related to quorum sensing has been reported on the clinical and physiological aspects of microorganisms at a molecular level, very little is known about the role of quorum sensing in the context of food, food processing, and food safety (Smith et al., 2004, Lu et al., 2004). Recently, Lu et al., (2004) demonstrated that certain food products contain compounds, which can inhibit the cell-cell communication. We are yet to understand how these inhibitory compounds affect bacterial processes in foods.

Quorum sensing was first described as a function of luminescence in marine bacteria, *Vibrio fischeri* and *Vibrio harveyi*. Both these organisms use autoinducer molecules for the regulation of bioluminescence. In both species, enzymes required for the production of light are coded by the luciferase structural operon *luxCDABE* (Miller et al., 2001)**.** *V. fischeri* proteins termed LuxI and LuxR are responsible for the quorum sensing system (Miller et al., 2001). In this pathway, LuxI produces and releases AI-1 molecules. When released, the AI-1 molecules accumulate to sufficient level, at which time they bind to the LuxR protein and finally activate the transcription of the *luxCDABE* operon. *Pseudomonas aeruginosa*, *Agrobacterium tumefaciens,* and *Erwinia carotowara* are some other bacterial species known to possess a quorum sensing system similar to that found in *V. fischeri*. Unlike *V. fischeri*, quorum sensing in *V. harveyi* consists of two-component circuit. In addition to AI-1 molecules, AI-2 molecules are also involved in the regulation of the bioluminescence. *V. harveyi* uses LuxI instead of LuxLM in the production of AI-1 molecules. Even though the proteins used for the production of AI-1 molecules are different in *V. fischeri* and *V. harveyi*, the pathways

appear to be identical (Bassler et al., 1993). The LuxS protein is responsible for the production of AI-2 molecules in the second component of quorum sensing system of *V. harveyi*. LuxN and LuxQ are generically similar sensors in *V. harveyi*, which can detect autoinducer molecules AI-1 and AI-2, respectively. The protein LuxP, in conjunction with LuxQ, transduces AI-2 molecules produced from LuxS to the sensor LuxQ (Miller et al., 2001). Many bacterial species are known to possess LuxS genes for the production of AI-2 molecules (Sperandio et al., 2001). AI-2 molecules are thought to be employed for interspecies communication. The pathway for the production of AI-2 molecules is almost identical in *V. harveyi* and other bacterial species (Smith et al., 2004; Miller et al., 2001). Many Gram (-) bacterial species can produce AI-2-like molecules and the maximum production for these AI-2-like molecules is at late logarithmic growth phase. Recently, Henke et al. (2004) demonstrated that *V. harveyi* contains a third regulatory cycle identical to that found in *V. cholerae*. This system consists of a CqsA- dependent autoinducer(CAI-1) and sensor CqsS. Several other vibrio species are known to contain the system for CAI-1 production. It is still unknown whether *E. coli*, *Salmonella* or any other enteric bacterial species have genetic elements that are homologous to the CAI-1. series of enzymatic steps and yields different products in addition to AI-2 molecule such as homocysteine and 4, 5- Dihydroxy- 2, 3- pentanedione.

The pathway for the biosynthesis of AI-2-like molecules from methionine is as follows (Figure 1). (Schaunder et al., 2001; Zhao et al., 2003;Winzer et al., 2003).

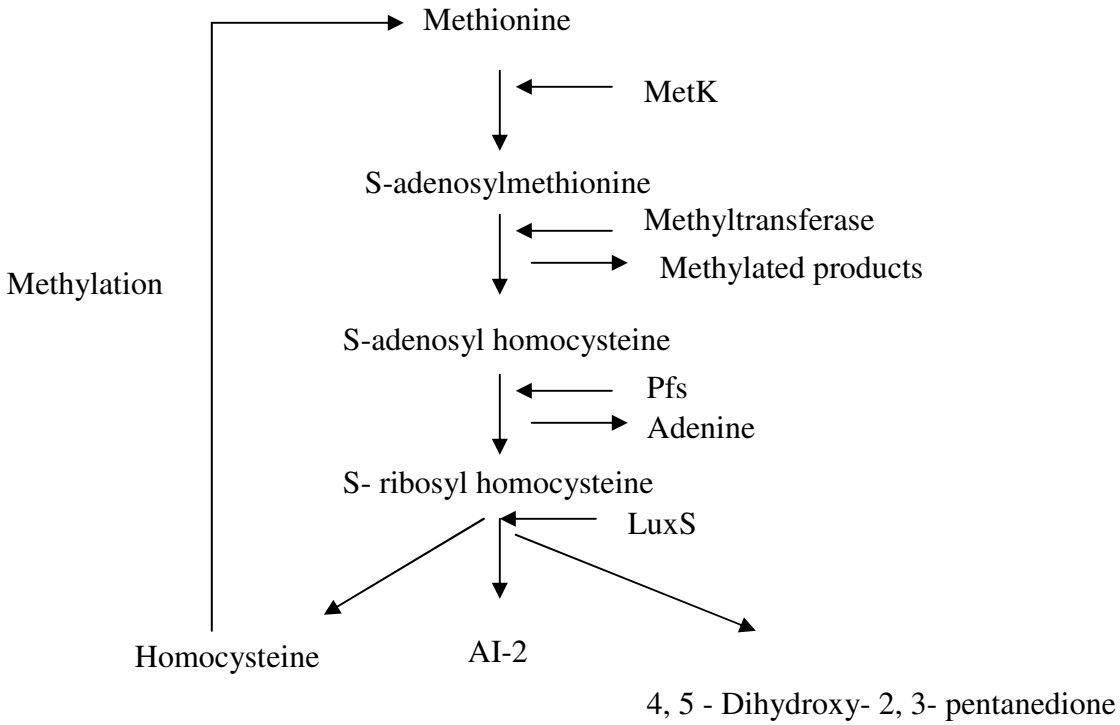

FIGURE 1. Pathway for the biosynthesis of AI-2 molecules.

Winzer et al. (2003) have also described the pathway for synthesis of AI-2 molecules and according to them AI-2 is not a signaling molecule, but it is a metabolite which is released in the early stage and metabolized in the later stage of cell cycle. Gram-positive bacteria also use small peptides as primary molecules in addition to AI-2 for the quorum sensing cascade (Smith et al., 2004; Miller et al., 2001). In this system, the peptide signal forms and releases from the precursor protein (Miller et al., 2001; Smith et al., 2004). Released peptide molecules are transferred and accumulate outside

of the cell via a transport mechanism. When these peptide molecules attain a threshold level, they initiate a series of phosphorylation/autophosphorylation steps that ultimately control global gene expression. Examples of these peptides are competence-stimulating peptides (CSP) in *Streptococcus pneumoniae*, competence and sporulation factor (CSF), ComX in *Bacillus subtilis*, and RNAIII in *Staphylococcus aureus* (Miller et al., 2001; Smith et al., 2004).

QUORUM SENSING IN *E. coli*

The quorum sensing system of *E. col*i O157:H7 is regulated through AI-2 molecules synthesized by LuxS protein. *E. coli* cells do not synthesize AI-1 molecules. *E. coli* consists of LuxR homologue, known as SdiA, but lacks the homologue for LuxI (Smith et al., 2004). Although *E. coli* O157:H7 has the structural components to recognize AI-1 molecules, it does not have components to produce AI-1 molecules (Michael et al., 2001). The quorum sensing system of S*almonella* is identical to that found in *E. coli* in that it does not have the genetic element necessary for the production of AI-1 molecules (Smith et al., 2003; Michael et al., 2001).

The quorum sensing pathway in *E. coli* O157:H7 is important in the regulation of virulence genes located in the LEE region. The genes located in the LEE region are responsible for encoding the attaching and effacing lesion, which causes the typical histopathology in large intestinal cells (Sperandio et al., 1999). *E. coli* O157:H7 causes a characteristic histopathology in large intestinal epithetical cells known as attaching and effacing. The LEE region is divided in five major polycistronic operons named as LEE1 through *LEE4* and *tir* (Sperandio et al., 1999). The first gene in the *LEE1* operon is *ler*

whose product activates *LEE2, LEE3,* and the *tir* operon, but not *LEE4*, in the presence of preconditioned cell free supernatant containing AI-2-like activity (Sperandio et al., 1999). QseA, a putative regulator of the LysR family, activates the transcription of *ler* and, therefore, other LEE regions. Mutations in *qseA* or *ler* significantly reduce typeIII secretion activity (Sperandio et al., 2002).

Quorum sensing in *E. coli* O157:H7 is also involved in the regulation of the genes controlling flagellar action and motility. The two-component regulatory system, called QseBC, is known to control the genes for flagella action and motility (Sperandio et al., 2002). In this regulatory system, *qseB* encodes for the response regulator, while *qseC* encodes for the sensor kinase. The quorum sensing molecules are involved in the regulation of *qseB.* Sperandio et al. (2001) demonstrated that the regulation of 404 (10% of total) genes of *E. coli* O157:H7 strain 86-24 in the presence of AI-2-like activity, while Delisa et al. (2001) demonstrated that the regulation of 242 (5.6% of total) genes of *E. coli* O157:H7 strain MDAI2 in the presence of AI-2-like activity. The difference observed in the percentage of genes regulated in the presence of AI-2-like molecules between two labs could be the result of difference in strain, media, and the procedures used. The quorum sensing system mediated by AI-2-like molecules can also promote an antibiotic resistance development in bacteria. Lu et al. (2004) have shown the transfer of *tet*(A) gene from tetracycline resistant strain to sensitive strain of *E. coli* in the presence of AI-2-like molecules (unpublished data). Recently, Sperandio et al. (2003) demonstrated that rather than AI-2, AI-3 molecules are responsible for the control of genes involved in the motility, flagella and typeIII secretion system. According to them,

the *luxS* gene is also involved in the production of AI-3 molecules. Sperandio et al. (2003) have shown that *invitro* synthesized AI-2 molecules failed to activate the expression of the LEE genes involved in motility and flagella. Furthermore, they have shown that, unlike AI-2, the cell fraction (AI-3) which binds to the C-18 Sep Pack columns and extracts with methanol is responsible for the activation of the LEE region and QseBC system. Epinephrine can also activate the LEE region in *E. coli* O157:H7 suggesting that epinephrine and AI-3 work along a common pathway for the activation of LEE region. Epinephrine is now considered involved in the bacterial cell-host communication (Sperandio et al., 2003).

FOOD BORNE DISEASES IN THE UNITED STATES

Food borne diseases cause about 76 million illnesses, 325,000 hospitalizations, and approximately 5000 deaths each year in the United States (Mead et al., 1999). Symptoms of food borne illnesses include nausea, vomiting, abdominal cramps, diarrhea, gastroenteritis, renal and hepatic disorder, and neurological syndromes. *Salmonella* (31%), *Listeria* (28%), *Toxoplasma* (21%), Norwalk-like viruses (7%), *Campylobacter* (5%) and *E. coli* O157:H7 (3%) are the major contributors to food borne deaths (Mead et al., 1999). Data published by Mead et al. (1999) states that of the 76 million illnesses, each year 38.6 million illnesses were caused by the known pathogens including 5.2 million (13%) due to bacteria, 2.5 million (7%) due to parasites, and 30.9 million (80%) due to viruses. Out of the total food-related incidences, *E. coli* O157:H7 causes about 62,458 (0.5%) illnesses, 2,168 (3%) hospitalizations, and 61(2.9%) death each year (Mead et al., 1999). A national health objective of reducing the major bacterial

food borne illnesses is intended to be achieved by the end of 2010 [(objectives 10-1a to 10-1d); U.S. Department of Health and Human service, 2000]. Data collected by the Centers for Disease Control and Prevention (CDC) in 2002 and 2003 showed that there was a significant decrease in the major food borne illnesses related to *Campylobacter*, *Listeria*, and *Salmonella* compared to data obtained during 1996-2001 (MMWR, 2003). Furthermore the Morbidity and Mortality Weekly Report (MMWR) data (2003 and 2004) did not indicate a sustained decrease in the food borne illness related to *E. coli* O157:H7 and some of *Salmonella* species in 2002, however, they did find a sustained decline in 2003. The decline observed in *E. coli* O157:H7 and *Salmonella* during 2003 could be due to an obvious limitation associated with the underestimation of cases associated with this organism. These data posses the question whether available intervention strategies to control *E. coli* O157:H7 and *Salmonella* are as effective as? Compared to *Salmonella*, *E. coli* O157:H7 has fewer incidences of food borne illnesses, but the infective dose is low compare to *Salmonella*, which makes it of serious food safety risk. Until recently, only ground beef and other meat products were identified as sources of *E. coli* O157:H7, however, recently apple cidar, alfalfa, and some other products have also be shown to be involved in *E. coli* O157:H7 infections. Data obtained by CDC in 2002 and 2003 were based on the laboratory-diagnosed, thus it is probably biased by factors that affect the probability of an illness being reported. Efforts to reduce the rate of food borne illness associated with high-risk food safety pathogens should be targeted to high-risk foods pro-actively rather than attempting to treat infected persons. Thus, effective pathogen intervention strategies are critically important to combat food

borne diseases. Since quorum sensing is thought to control the expression of several microbial genes, the use of quorum sensing-based pathogen-intervention strategies may have significant potential in near future.

E. coli O157:H7 AND FOOD BORNE DISEASE

E. coli O157:H7 is a commensal organism of bovine animals and ground beef and milk products serve as major vehicles for its transfer to humans (Griffin et al., 1991). *E. coli* O157:H7 is a causative agent of hemolytic uremic syndrome (HUS), hemorrhagic colitis (HS), and thrombocytopenic purpura (TTP). Symptoms for HS include bloody diarrhea and stomach cramps. Similarly, HUS causes bloody diarrhea, acute renal failure, and kidney failure (Ruggenenti et al., 1998). Furthermore, TTP has been shown to result in neurological disorders and kidney failure (Ruggenenti et al., 1998). *E. coli* O157:H7 has been associated with a number of food borne outbreaks (Hudson et al., 1997; Stewart et al., 1983). *E. coli* O157:H7 causes a characteristic histopathology in large intestinal epithetical cells known as attaching and effacing. The LEE region (locus of enterocyte effacement) is a pathogenicity island that encodes the protein involved in the formation of attaching and effacing lesions (Sperandio et al., 2002). Since 1982, food borne outbreaks of *E. coli* O157:H7 have occurred in several states in the U.S (Bean et al., 1996). A variety of other foods, such as fresh cut salad, apple cider, and watermelon have also been involved in outbreaks of *E. coli* O157:H7 (Brackett et al., 1994; Mead et al., 1999; Riley et al., 1983). Human illnesses due to *E. coli* O157:H7 are mainly related to the consumption of food or water that have been contaminated with *E. coli* O157:H7 containing bovine feces. The ability of *E. coli*

O157:H7 to survive in a low pH environment and refrigerated products makes it of concern, because the infective dose for this organism is very low (Spika et al., 1986). During 2003, 6584 samples of ground beef products were analyzed and 20 (0.3%) of those samples were found to be positive for *E. coli* O157:H7 (FSIS, 2004). Human infection from *E. coli* O157:H7 declined in 2003, which otherwise was stagnant between 1996-2002 (MMWR, 2004). This is probably the result of an October 2002 FSIS notice to manufacturers of raw ground beef products that they must reassess their HACCP plans regarding this pathogen (MMWR, 2004). Presently, beef processing plants do not distribute raw ground beef lots unless microbiological tests performed at the plant are negative for *E. coli* O157 (MMWR, 2004).

CONTROL OF BACTERIAL PROCESSES USING QUORUM SENSING-BASED APPROACHES

In recent years, the efficiency of antibiotics against infections is decreasing, because of the increased antibiotic resistance in many bacteria (Raffa et al., 2004). Furthermore, antibiotics are less effective against microbial biofilms as biofilm-associated bacteria are typically 1000-fold more resistant to antibiotics compared to free-living bacteria (Stoodely et al., 2003). To overcome these problems, existing techniques must be replaced or modified to control various bacterial processes. It is believed that by suppressing cell-cell communication we can control various undesirable bacterial processes such as virulence gene expression, sporulation, and survival (Pillai et al., 2004). Development of an intervention technologies based on interfering cell-cell communication disturbance will be a novel approach to control the various bacterial

processes, which otherwise would be difficult or impossible to control using current approaches (Raffa et al., 2004; Pillai et al., 2004). Compounds such as furanones, S-anhydroribosyl-L-homocystein, and S-homoribosyl-L-cysteine have been chemically synthesized and can serve as antiquorum sensing molecules (Alfaro et al., 2004; Wu et al., 2004). Interestingly, some foods, especially meat products including ground beef, contain compounds that can inhibit AI-2-like activity (Lu et al., 2004). Others have suggested that the effects of quorum sensing inhibitory compounds in controlling various bacterial processes may lead to the development of novel intervention strategies and in drug formulation. Acqua et al. (2004) demonstrated that the disruption of cell-cell communication in *Staphylococcus* infection using RNAIII-inhabiting peptide (RIP). Wu et al. (2004) demonstrated that synthetic furanones inhibit bacterial quorum sensing and enhances clearance of *Pseudomonas aeruginosa* lung. Also, Tateda et al. (2005) and Tomono (2005) demonstrated that macrolides can be used in controlling chronic *P. aeruginosa* infection by inhibiting cell-cell signaling. Ren et al. (2004) demonstrated, using DNA microarray-based analysis, that brominated furanones interfere with AI-2 bacterial signaling. The presence of brominated furanones at a level of 100 µg/ml resulted in a two-fold decrease in autoinducer activity. On the other hand, the inhibitory compounds found in meats exhibited considerable inhibitory action (about 90%) on the expression of AI-2-like activity (Lu et al., 2004). It may be very important in the future to identify and purify inhibitory compounds present in foods for the development of novel strategies to safeguard food products from undesirable microbial activities.

QUORUM SENSING IN RELATION TO FOODS

Control of spoilage and food borne illnesses associated with microorganisms in foods is one of the biggest challenges facing the food industry. Efforts have been made to control these problems using available technologies, but it seems difficult or impossible using available conventional approaches. Since cell-cell signaling is believed to control many bacterial processes, interfering with this process possibly could solve some of the major problems associated with controlling pathogens in foods (Lu et al., 2004; Pillai 2004., Smith et al., 2004). Presently, our knowledge is very limited concerning the role of quorum sensing molecules and bacterial activity in food systems and how this signaling process affects spoilage, pathogenicity, growth, antibiotic resistance, biofilm formation, and survival in foods. An understanding of the role of quorum sensing molecules present in foods could aid in the formulation of new strategies to control pathogen survival and proliferation in foods. Quorum sensing is likely occuring in both spoilage and pathogenic bacterial species(Smith et al., 2004). Christensen et al. (2003) demonstrated the role of *S. proteamaculans* in milk curdling using a quorum sensing system. The AHL molecules produced by *S. proteamaculans* seem to be responsible for milk curdling. Furthermore Bruhn et al. (2004) have shown that *Serratia Proteamaculans* can also utilize the AHL (AI-1) molecules produced by *Hafina alvei*, which is the most commonly identified AHL-producing bacterium in the food system. Interestingly, the AHL produced by *H. alvei* were able to cause milk spoilage but they failed to show any effect by themselves in meat spoilage (Bruhn et al., 2004). These interfering results supports the necessity of studying the behavior of

bacterial processes in context of food systems as difference in food systems can also lead the difference in bacterial processes. Spoilage of food products occurs usually at high number of microflora (10^8-10^9 CFU/gm) and the effects of quorum sensing molecules also occur usually at high bacterial numbers (10^7-10^8) (Gram et al., 1999). Cloak et al. (2002) studied the production of AI-2-like activity in food systems such as milk, chicken broth, and apple juice by organisms such as *E. coli* O157:H7, *S*. Typhimurium, C. *jejuni*, and C. *coli*. According to their results, *E. coli* O157:H7 and *S*. Typhimurium can produce AI-2-like activity even at 4°C in milk and chicken broth within 3 hrs. Intracellular metabolism and stress conditions can alter the AI-2 production pattern in *E. coli* K-12 (Delisa et al., 2001). *S*. Typhimurium cannot grow at 4°C, but the reason for the production of AI-2-like activity could be stress or change in metabolic conditions. The production of AI-2-like activity at 4°C shows that if quorum sensing is involved in the regulation of various bacterial processes in food systems, then our traditional food preservation systems such as refrigeration may not be actually safe. C. *jejuni* and C. *coli* have also exhibited the production of AI-2-like activity, but only at 37°C. Furthermore, in apple juice Clock et al. (2002) did not observe any AI-2-like activity when subjected with different microorganisms in different growth conditions (time/temp). Other food items such as tomato, carrot, cantaloupe, fish, and tofu have been shown to possess AI-2-like activity (Lu et al., 2004). In addition, Lu et al. (2004) studied the effect of different food preservatives such as sodium acetate, benzoic acid, propionic acid, and sodium nitrate on AI-2-like activity. Their results showed that the presence of these food preservative compounds inhibited the expression of AI-2-like activity about 75% to

99%, suggesting that these chemicals were interfering the cell-cell communications. *Erwinia carotovora* is known to produce soft rot in different food items such as fruits, vegetables, and potatoes. Quorum sensing molecules favors the formation of soft rot through the upregulation of the production of some of the exoenzymes (Smadja et al., 2004; Toth et al., 2004). Today, we have very limited information about role of quorum sensing molecules in food systems and we need to expand our current limited knowledge of quorum sensing in food systems.

IMPORTANCE OF *hha* GENE

In *E. coli*, synthesis and secretion of the hemolysin are determined by the *hly* operon, while the *hha* gene is involved in the production of the hemolysin regulating protein (Jubete at al., 1995). Hemolysin is the cell product that can increase virulence of *E. coli* strain (Brauner et al., 1995). The genes involved in hemolysin are located in the *hly* region. *hlyR* is a 600 bp sequence located 1.5 kb upstream of the gene *hlyC* of the *hly* region (Jubete at al., 1995). The absence of the *hlyR* region causes cells to show poor hemolysin expression (Goebal et al., 1982). The hemolysin modulating effect of *hha* is mediated by possible interaction with a 220-bp sequence located in *hylC* named *hylM* region (Jubete at al., 1995). Mutations in the *hha* gene have been shown to produce higher gene expression *hly* by overcoming the silencing effect produced by its interaction with *hylM* region (Jubete at al., 1995). Furthermore, hemolysin expression is also modulated by environmental factors such as osmolarity, temperature, and anaerobiosis (Mourino et al., 1996; Mourino et al., 1998). Hha protein plays a role both in osmolarity and temperature-dependent modulation of hemolysin expression. Mutation

in the *hha* gene also leads to increased toxin (Vir) production (Mourino et al., 1996). The transcriptional activator *hilA* controls the transcription of pathogenicity island 1 in *Salmonella* invasive phenotypes (Fahlen et al; 2001). The expression of *hilA* is controlled by environmental factor such as pH, osmolarity, and growth phase. Variation in these factors leads to the repression of *hilA*. The *hha* gene is also a negative regulator for the transcriptional activator *hilA* (Fahlen et al; 2001). The first gene of *LEE1* operon, *ler*, is a positive regulator for the operon *LEE2*, *LEE3* and *tir* of LEE region (Sperandio et al., 1999). Recently, Sharma et al. (2004) demonstrated that binding of *ler* to the *esp* promoter also regulates the transcription of the *esp* operon (LEE4). The mutation in the *hha* gene is seen as an increased transcription of the *ler* and *esp*, while the wild type strain containing *hha* gene mediates the repression of *ler*, which causes the reduced expression of *esp* operon as well (Sharma et al., 2004). Therefore, *hha* also serve as a negative regulator of LEE region. Furthermore, Hha protein shows homology with the YmoA protein of *Yersinia enterocolitica*. The YmoA protein is a temperature-dependant modulator of the expression of virulence factor in *Yersinia* (Carmona et al., 1993, and Cruz et al., 1992) and *hha* gene can complement the phenotype of *YmoA* mutant (Milkuskis et al., 1994).

The *yad*K gene is involved in the functions such as mobility, secretion, and adhesion (Welch et al., 2002). We did not find much specific information on it during the literature search conducted on 24th Jan 2004.

MATERIAL AND METHODS

BACTERIAL STRAINS

Two different strains of *E. coli* O157:H7, namely ATCC 43895 and VS 94, were used in the survival and virulence gene expression studies. ATCC 43895 is a wild type strain that can produce and sense AI-2-like molecules in the presence of glucose. VS-94 is a *luxS* mutant strain, which cannot produce AI-2-like molecules, but can sense externally added or produced AI-2-like molecules (Sperandio et al., 2001). VS 94, an isogenic mutant of *E. coli* O157:H7 strain 86-24 and resistant to tetracycline (10 µg/ml) was kindly provided by Vanessa Sperandio. The reporter strain V. *harveyi* BB170 (*luxN*::Tn5 sensor 1^- sensor 2^+), which can only sense autoinducer-2 (AI-2) activity was kindly provided by B. L. Bassler (Princeton University) and was used for autoinducer activity bioassay. Luria-Bertani (LB) broth medium supplemented with 0.5% of glucose(unless noted otherwise) was used to culture *E. coli* O157:H7. The V. *harveyi* BB170 cells were grown in the autoinducer bioassay (AB medium) (Surette et al., 1998). *E. coli* O157:H7 cells were grown at 37˚C while V. *harveyi* cells were grown at 30˚C with shaking and aeration.

MICROBIOLOGICAL MEDIA

The autoinducer bioassay (AB) medium was prepared as follows (Lu et al., 2004). A solution consisting of NaCl (17.5 g/liter; Sigma, St. Louis, MO), $MgSO_4$ (12.3 g/liter; Fischer Scientific, Fair Lawn, NJ) and casmino acid (2 g/liter; Fischer Scientifics) was adjusted to pH 7.5 and sterilized by autoclaving (15 min, 121˚C, 15 psi). After autoclaving, the solution was allowed to cool to room temperature and sterilize 1 M potassium phosphate (pH 7.0, 10 ml/liter; EMD chemicals, Gibbstown, NJ), 50% sterile glycerol (20 ml/liter; EM science, Gibbstown, N.J), and filter sterilized 0.1 M L–arginine (10 ml/liter; Sigma) were added to it.

PREPARATION OF CELL-FREE SUPERNATANT

Cell free supernatant (CFS) of *E. coli* O157:H7 that was freshly prepared or stored at -20˚C was used as an exogenous source of AI-2-like molecules and was designated as "Preformed Cell Free Supernatant" (PCFS). The preparation of PCFS was as follows: Briefly, an overnight culture of ATCC 43895 was inoculated in fresh LB broth supplemented with 0.5% glucose and grown approximately to an OD_{600nm} of 1.2. At this time, the cell pellets were collected by centrifugation (3,313× *g* for 5 min) and supernatant was filtered using 0.22-µm filters and stored at –20˚C until used. The PCFS was checked for AI-2-like activity before being used in experiments. Heat treatment has been shown to destroy AI-2-like activity (Surette et al., 1998; Lu et al., unpublished data). AI-2-like activity of PCFS could be destroyed by autoclaving (15 min, 121˚C, 15 psi), such treated CFS were termed as "Autoclaved Cell Free Supernatant" (ACFS). The

destruction of AI-2-like activity in ACFS was confirmed using AI-2 activity bioassay and the preparation was stored at -20˚ C until use.

PREPARATION OF THE GROUND BEEF EXTRACTS

Ground beef patties were purchased from the Texas A&M University Rosenthal retail meat center, stored at -20˚ C, and thawed at room temperature for 2 hours before use. To prepare Uncooked ground beef extracts, ground beef patties were mixed (1:1, w/v) with 0.1 M sterile phosphate buffer in sterile Whirl-Pak bags (VBR International) and stomached for 2 min. After stomaching, the preparation was centrifuged at 6000× *g* for 10 min at room temperature. The supernatant was filtered through Whatman filter paper (no 2) followed by 0.2 µm pore size vacuum filtration (Stericup, Micropore Corp, Bedford, MA, USA). To prepare cooked ground beef extract, the ground beef patties were stomached in sterile Whirl-Pak bags and transferred to 45-ml sterile plastic tubes. The plastic tubes were placed in a water bath to achieve an internal time-temperature combination of 67˚C for 41 sec (Section- 318.23, office of federal register, code of federal regulations, Title-9, Part 200 to end, US govt printing office, Washington-2002). To prepare "autoclaved ground beef extracts", ground beef patties were stomached in sterile Whirl-Pak bags, transferred to sterile glass beakers, and heat-treated at time-temperature combination of 121˚C for 15 min at a 15 psi in an autoclave. Cooked and autoclaved beef extracts were mixed with phosphate buffer (0.1 M), filtered, and centrifuged as indicated above. If needed, the cooked and autoclaved ground beef extracts were warmed at 37˚C to facilitate filtration. The ground beef extracts were stored at 4˚C before use.

AI-2 ACTIVITY BIOASSAY

The reporter strain *V. harveyi* BB170 (*luxN*::Tn5 sensor 1^- sensor 2^+), which exhibits the luminescence response only in the presence of AI-2 molecules, was used to determine the AI-2-like activity (Surette et al., 1998). The operon *lux*CDABE in this strains encodes for the luminescence response in the presence of AI-2 molecules, because of binding between AI-2 molecules and sensor-2. The bioassay used was similar to that reported by Surette and Bassler (1998) and Lu et al. (2004). The AI-2 bioassay was as follows: Briefly, an overnight grown culture (approximately 10^8 CFU/ml) of *V. harveyi* BB170 was inoculated (1:5000) into AB media. Ninety microliters of the freshly diluted cultures were mixed with 10 µl of test samples in a 96-well micro plate (Perkin Elmer Life Science Inc., Boston, Mass). The PCFS was used as a positive control, while AB media was used as a negative control. The micro plate was incubated at 30˚C with shaking at 121 rpm and the luminescence response was (Perkin Elmer Wallac Victor-2 luminometer) measured every 60 min for the initial 3 hours and there after every 30 min. The end of experiment was noted when there was a significant change in the luminance response of the negative control. The data were recorded as “relative AI-2-like activity”, which was calculated as the ratio of luminescence of the test sample to that of the negative control.

AI-2-LIKE ACTIVITY IN *E. coli* O157:H7 STRAINS

AI-2-like activity of the wild type strain ATCC 43895 and the mutant strain VS 94 were measured to confirm AI-2-like activity production in wild type strain ATCC 43895 and absence of AI-2-like activity in mutant strain VS94.

An overnight culture of ATCC 43895, VS94 were inoculated (1:100) in separate flask containing fresh LB broth supplemented with 0.5% glucose, and grown aerobically. Growth was measured using optical density (OD_{600nm}) readings. The cell free supernatants (CFS) were prepared by removing 1 ml of the aerobically growing culture, centrifuging for 1 min (18,000 × *g* at room temperature), and passing the supernatant through a 0.2 µm syringe filter (Corning, New York, N.Y). The OD_{600nm} reading and CFS were collected at one-hour time intervals for eight hours and at the end of 24 hours. The CFS were stored at -20 ˚ C until used for the AI-2 bioassay.

HEAT LABILITY OF INHIBITORY COMPOUNDS FROM MEAT

The inhibitory effects of ground beef extracts on AI-2-like activity was measured as mentioned previously by Lu et al. (2004). Results were however expressed as relative AI-2-like activity. Cooked and autoclaved ground beef extracts were used in AI-2 bioassay to measure the heat lability of inhibitory compounds. Different proportions of ground beef extracts and PCFS were mixed in the wells of the micro plate with 90 µl of the freshly diluted culture of V. *harveyi* BB 170 in order to measure the amount of AI-2-like molecules needed to overcome inhibitory effects. The different proportion includes 1 µl of ground beef extracts plus 9 µl of PCFS, 2 µl of ground beef extracts plus 8 µl of PCFS, and up to 9 µl of ground beef extracts plus 1 µl of PCFS. The

positive control consisted of 10 µl of PCFS instead of ground beef extracts while the AB media was used as a negative control.

SURVIVAL OF *E. coli* O157:H7 IN GROUND BEEF EXTRACTS

The survival study of *E. coli* O157:H7 in the different ground beef treatment extracts was studied under refrigeration conditions (4˚C). Two different strains of *E. coli* O157:H7, ATCC 43895 and VS 94, were used in the survival study. The PCFS, previously obtained from logarithmic phase cultures of strain ATCC 43895, was used as a source of exogenous AI-2-like molecules. ACFS, which was autoclaved to destroy AI-2-like activity, and phosphate buffer (0.1 M) were used as a negative control. The ground beef extracts (uncooked, cooked, and autoclaved) were each mixed in sterile glass test tubes with ACFS, and phosphate buffer in the proportion of 1:1 (5 ml each). The samples were inoculated with washed and serially diluted *E. coli* O157:H7 cells to give a final inoculum level of ~ 10^4 CFU/ml. The cells were washed as follows: 0.1 M phosphate buffer was used in the equal amount of culture broth to wash the *E. coli* O157:H7 cells pellets obtained during logarithmic phase growth. The cells were resuspended in phosphate buffer and centrifuge at 3,313× *g* for 5 min. This washing step was repeated for three times before the cells were used as an inoculum. The glass test tubes were covered with parafilm, capped and incubated at 4˚C and cell numbers (CFU/ml) were determined by serial dilution in phosphate buffer (0.1 M) at time intervals of 0, 1, 5, 10, 15, 20, 25, and 30 days by plating on Luria- Bertani (LB) agar. The experiment treatments were prepared in triplicate tubes and each tube was used for

serial dilution. The LB plates for VS 94 were amended with tetracycline (10 µg/ml). The LB plates were incubated at 37˚C for 18 hrs prior to enumeration.

MEASUREMENT OF *hha* AND *yadK* GENE EXPRESSION IN *E. coli* O157:H7 IN GROUND BEEF EXTRACTS

Changes in gene expression of the two selected genes in *E. coli* O157:H7 were studied using real-time PCR analysis. The *hha* and *yadK* genes of *E. coli* O157:H7 were targeted to determine the effect of AI-2-like activity on gene expression in the presence of ground beef extracts. The *hha* gene modulates hemolysis expression, while the *yadK* gene is involved in the production of putative fimbrial like protein in *E. coli* O157:H7. The 16S rRNA gene was used for the normalization of the gene expression data. The method used by Delisa et al. (2001) for the treatment of the cells in the presence of AI-2-like molecules was employed with slight modification.

Overnight cultures of *E. coli* O157:H7 (ATCC 43895 and VS 94) were inoculated seperately (1 % [vol/vol] inoculums) in fresh LB broth supplemented with 0.5% of glucose and grown aerobically to 1.0 OD_{600nm}. Meanwhile, 1.5 ml of ground beef extracts (uncooked, cooked and autoclaved) were mixed with 1.5 ml of PCFS or LB broth and raised to a temperature of 37˚ C in water bath. Once the logarithmic phase grown cultures attained approximately 1.0 OD_{600nm}, they were divided into 3 ml aliquots in seperate tubes and centrifuged for 5 min (3,313 × *g* at 4˚C) followed by complete removal of the supernatant. The cell pellets of *luxS* mutant strain VS94 were resuspended with 3 ml mixture of ground beef extracts and PCFS or LB broth. The cell pellets of ATCC 43895 were resuspended in a mixture of ground beef extracts and

PCFS. The resuspended cells were incubated for 25 min of aerobic growth conditions in a shaking incubator (37˚C). The resuspended cell mixtures were centrifuged for 5 min (3,313 × *g* at 4˚C) and the bacterial pellets were collected after removal of the supernatant. The bacterial pellets were used immediately for RNA extraction or flash frozen in liquid nitrogen and stored at -80˚C before use. Total RNA was extracted by the method prescribed by the manufactures (Ribopure ^{TM} - Bacteria; Ambion Inc., Austin, TX) and was used in cDNA synthesis and real time PCR anaysis. The extracted RNA was stored at -80˚C until used for cDNA synthesis. The synthesis of cDNA was performed using the Gene Amp® RNA PCR Kit (Applied Biosystems Branchburg, NJ). The cDNA was stored at -20˚C before use in the real-time PCR analysis. The procedure for RNA extraction, cDNA synthesis and real-time PCR were as follows:

cDNA SYNTHESIS

cDNA synthesizing reagents were obtained from Gene Amp® RNA PCR Kit (Applied Biosystems, Branchburg, NJ). The reaction mixture was as follows: The reaction utilized 1 µl of RNA template and 19 µl of a master mix consisting of 4 µl of 25 mM $Mgcl_2$ solution, 2 µl of 10 X PCR buffer, 2 µl of DEPC treated DI water, 2 µl of dATP, 2 µl of dTTP, 2 µl of dGTP, 2 µl of dCTP, 1 µl of RNase inhibitor, 1 µl of MuLV reverse transcriptase enzyme, and 1 µl of random hexamer. The reaction mixture was incubated for 10 min at room temperature before placement in the thermocycler. First strand synthesis of cDNA was carried out using single cycle Reverse-Transcriptase polymerase chain reaction (RT-PCR). The RT- PCR cycle conditions were as follows: 60 min at 42˚ C for annealing and elongation followed by 5 min at 99˚C to inactivate the

reverse transcriptase enzyme. All RT-PCR cycle steps were performed using programmable thermocycler (Applied Biosystems, Gene Amp* PCR system 2700). The cDNA samples were stored at -20˚ C until used.

REAL-TIME PCR AMPLIFICATION FOR QUANTITATIVE GENE EXPRESSION ANALYSIS

The cDNA stored at -20˚ C or freshly prepared cDNA was used as template in real-time PCR analysis. The primer sequence utilized for the specific targets are shown in (Table-1). The 384-well clear optical reaction plate (Applied Biosystems) was filled with 1 µl of cDNA as a template and 19 µl of the master mix. The master mix placed in individual wells were consisted of 10 µl of SYBR® GREEN PCR mix (Applied Biosystems, Warrington, UK), primers (0.6 µl [10 µmol] for *hha*-F, *hha*-R, *yadK*-F and *yadK*-R; while 0.9 µl [10 µmol] for *16S*-F and *16S*-R) and adjusted to 19 µl using DEPC-treated deionized water. The negative controls consisted of DEPC-treated DI water instead of cDNA. The plates were sealed using the optical adhesive covers (Applied Biosystems, Foster City, CA). The sealed plate was placed on a thermocycler (Applied Biosystems, Unit- Abi- Prism, 7000 HT, Foster City, CA) and the instrument was programmed for relative quantification to obtain a C_T value and dissociation curve. The amount of primer added in the master mix was based on primer optimization. The primer optimization was carried out using master mix containing different concentration of primers. The instrument was programmed for absolute quantification and optimum amount of primer concentration was selected from dissociation curve. The change in gene expression was calculated using the formula: Treatment 2/treatment 1 = $2^{\Delta\Delta CT}$,

Where $\Delta\Delta C_T = \Delta C_T$ for treatment 1- ΔC_T for treatment 2 and ΔC_T is the difference between the C_T value of the target gene and the normalization gene (16S) (Dorak et al).

DATA ANALYSIS

For the survival study, kinetic parameters were calculated from the survival numbers obtained at different time period. The slope for the death rate (μ_d) was calculated using the linear regression equation. The death time (t_d) and death rate (k_d) was calculated as shown by M. Bachrouri et al. (2002). Table -2 shows the linear regression equation, which is an average of three line equation and kinetic parameters from the death periods (μ', $t_{d,}$ k').

All experiments were performed in triplicates. The autoinducer-2-like activities were expressed as relative AI-2-like activity. The change in gene expression was calculated as folds increase or decrease in gene expression using the mathematical formula given above. The data obtained from the survival study were analyzed by SPSS at 95% confidence level. One-way ANOWA test was performed to analyze the data obtained from the survival study.

TABLE 1- PRIMER SEQUENCE FOR SELECTED GENES USED IN GENE EXPRESSION STUDIES.

Gene	Primer Sequence
hha-F	5'-ATAATGAACTGGCGGTATTTTACTCA-3'
hha-R	5'- GTCGTACAGTTTATTCATGGTCAATTC-3'
yadK-F	5'- AACGTCGGCATTGTGATTTT-3'
yadK-R	5'- TCCGTTCCTCGCAVGTTAA-3'
16S-F	5'- CCAGCAGCCGCGGTAAT-3'
16S-R	5'- TGCGCTTTACGCCCAGTAAT-3'

*F= Forward primer, R= Reverse Primer

RESULTS

AI-2-LIKE ACTIVITY IN WILD TYPE AND *luxS* MUTANT *E. coli* O157:H7 STRAINS

The presence of AI-2-like activity during the growth of wild type (ATCC 43895) and the *luxS* mutant strain (VS 94) of *E.coli* O157:H7 strains is shown in Figure 2. Maximal AI-2-like activity was observed in the wild type strain between 2 and 4 hours when grown in LB broth. The mutant strain showed extremely low levels of AI-2 like activity during the 24 hours of incubation. In the wild type strain during these incubation conditions, maximal AI-2 like activity corresponded to 1-1.5 OD_{600} units. The maximal AI-2-like activity produced in wild type strain was about 150-250 fold relative light unit (RLU) compared to negative control when measured using the *Vibrio harveyi* reporter strain bioassay. The AI-2 like activity decreased beyond the first 3 to 4 hours of incubation. Furthermore, as mentioned by Miller et al. (2001), a decrease in AI-2-like activity was observed during the stationary phase of growth. Compared to wild type, very low level of AI-2-like activity was observed during any growth period of the *luxS* mutant strain, VS94. The AI-2-like activity observed in *luxS* mutant strain was about 5-8 fold RLU compared to negative control. There was apparently no difference in the growth pattern (based on OD_{600nm} readings) of the mutant and wild type strains even though the mutant strain had a mutation in the *luxS* gene.

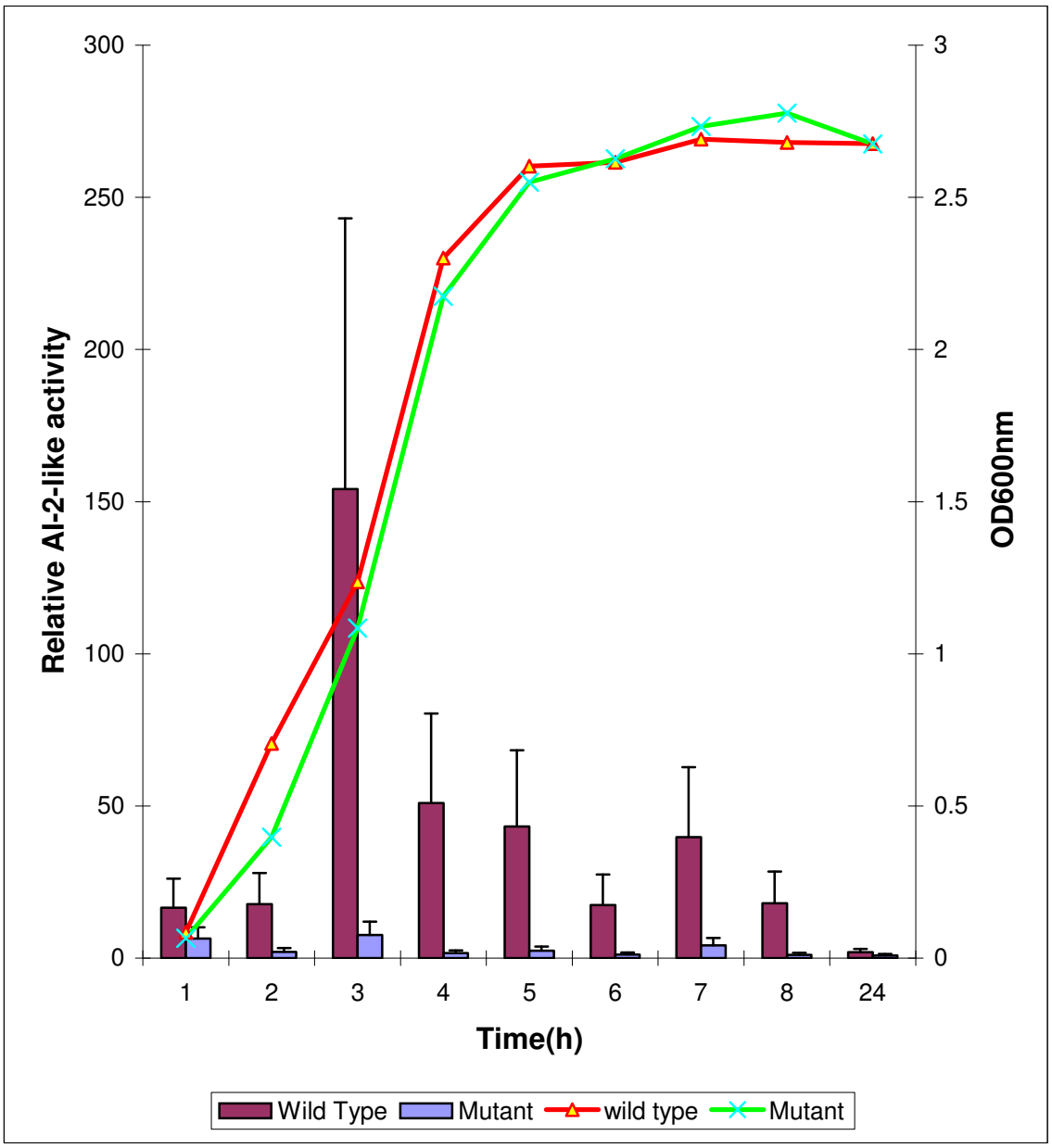

FIGURE 2. Relative AI-2-like activity of *E. coli* O157:H7 wild type and *luxS* mutant strains.

HEAT STABILITY OF INHIBITORY EFFECTS OF GROUND BEEF ON AI-2-LIKE ACTIVITY

The heat stability of the inhibitory effects of ground beef on AI-2 like activity is shown in Figure 3. In the presence of uncooked and cooked ground beef extracts, there were only minimal levels of AI-2 like activity as compared to the positive control. AI-2 like activity was observed only in the presence of autoclaved ground beef extracts. However, even in the presence of autoclaved ground beef extracts, it is evident that AI-2 like activity in the PCFS decreases with increasing amounts of ground beef in the AI-2 bioassay system. As the concentration of ground beef in the bioassay increased from 10% to 90% there was a corresponding decrease in AI-2 like activity. A relatively low amount of AI-2-like activity was observed in the PCFS when mixed with cooked and uncooked ground beef extracts as compared to the activity observed in the presence of autoclaved ground beef extracts. Mixing of 10%(v/v) of ground beef extracts with 90%(v/v) of PCFS resulted in almost the same amount of AI-2-like activity as shown in the positive control. Also the addition of 90% of PCFS to the 10% of cooked or uncooked ground beef extracts did not over come the inhibitory effects observed in the cooked and uncooked ground beef extracts.

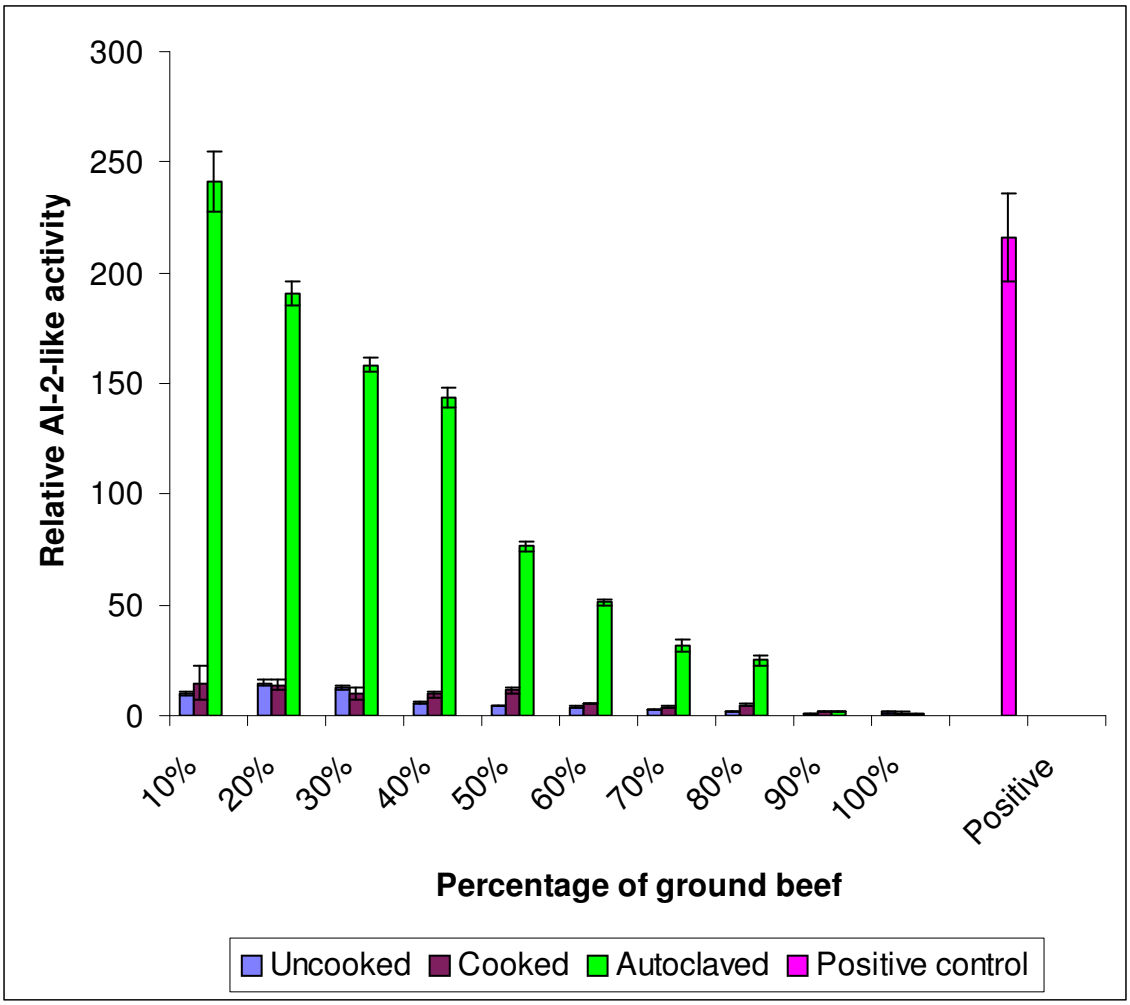

FIGURE 3. Measurement of AI-2-like activity in different types of ground beef extracts such as uncooked, cooked and autoclaved (100 % in figure). Different proportion of ground beef aliquots mixed with PCFS to measure the minimum amount of AI-2-like molecules needed to overcome the inhibition.

SURVIVAL OF *luxS* MUTANT AND WILD TYPE *E. coli* O157: H7 STRAINS AT 4°C IN THE PRESENCE OF PREFORMED CFS (PCFS), AUTOCLAVED CFS (ACFS), AND PHOSPHATE BUFFER (PB)

The survival of *E. coli* O157: H7 *luxS* mutant (VS 94) and wild type strain (ATCC 43895) in phosphate buffer at 4 ºC was studied in the presence of PCFS, ACFS, and phosphate buffer (PB) (Figure 4). The inactivation kinetics of the wild type and mutant strains is shown in Table 2. The ability of *luxS* mutant strain, VS94, to sense the externally added AI-2-like molecules and not to produce their own AI-2-like molecules, was used to determine the role of AI-2-like molecules on the survival of *E. coli* O157:H7. When PB was used as a suspending medium, the *luxS* mutant strain showed over a 2-log reduction by the end of the 30-days at 4º C compared to only a 1.4 log reduction that was observed in the wild type strain. This difference observed between the mutant and wild type strain was significant ($P < 0.05$). In the presence of ACFS (in which AI-2-like activity was destroyed by autoclaving), there was no significant difference ($P > 0.05$) between the wild type and the mutant strains. Approximately 1.3 log reduction was observed in both the wild type and mutant strains. No significant difference ($P > 0.05$) observed in the survival between wild type and mutant strain of *E. coli* O157: H7 when PCFS was used as the suspending medium. The survival of both strains were, however, significantly ($P < 0.05$) higher in PCFS than in ACFS. There was less than 1 log unit reduction in viable populations in the presence of PCFS as compared to the reduction observed in the presence of ACFS. Based on the inactivation kinetics, it is evident that the presence of PCFS had a protective effect on the survival of the *luxS*

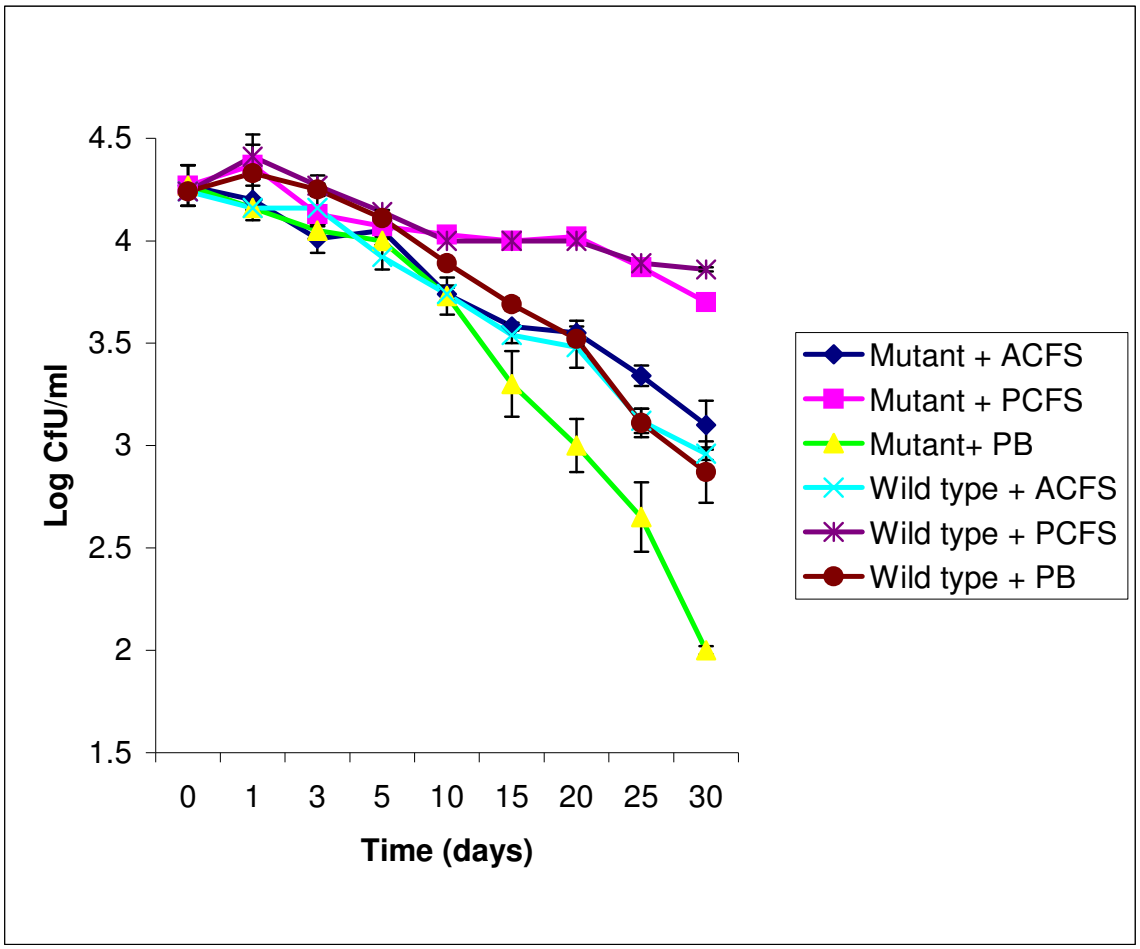

FIGURE 4. Survival of luxS mutant and wild type *E. coli* O157:H7 strain at 4C in the presence of preformed CFS, autoclaved CFS, and phosphate buffer.

TABLE 2 – INACTIVATION KINETICS OF *luxS* MUTANT (VS 94) AND WILD TYPE *E. coli* O157:H7 (ATCC 43895) AT 4°C IN THE PRESENCE OF PREFORMED CFS (PCFS), AUTOCLAVED CFS (ACFS), AND PHOSPHATE BUFFER (PB)

* Treatments[a]	[#] Regression equation[b]	r^2	$^{c}\mu_d$	$^{d}t_d$	$^{e}k_d$
W + ACFS	y= -0.042+4.21	0.9577	-0.042	-7.17	-0.14
W + PCFS	y= -0.016+4.28	0.7855	-0.016	-18.81	-0.05
W + PB	y= -0.047+4.35	0.9714	-0.047	-6.4	-0.16
M + ACFS	y= -0.036+4.19	0.9394	-0.036	-8.36	-0.12
M+ PCFS	y= -0.017+4.25	0.8113	-0.017	-17.71	-0.06
M + PB	y= -0.07+4.31	0.9566	-0.07	-4.3	-0.23

*W = Wild type; M = Mutant type; PB= Phosphate buffer; ACFS= Autoclaved cell free supernatant in which AI-2 like activity was destroyed; PCFS= Preformed cell free supernatant having AI-2 like activity; UCGB= uncooked ground beef extracts (raw); AGB= Autoclaved ground beef extracts; CGB= Cooked ground beef extracts

[#] Mean of three line equation and kinetic parameters from the death periods (μ', t_d, k')

$^{+}r^2$ = Pearson square coefficient; $^{c}\mu_d$ = Slope from the equation, $^{d}t_d$ = death time; $^{e}k_d$ = death rate

mutant strain as compared to the survival of the mutant in the presence of phosphate buffer and autoclaved CFS (ACFS) (Table 2). VS 94, the *luxS* mutant strain of *E. coli* O157: H7 is capable of sensing exogenous AI-2-like molecules, but incapable of producing its own AI-2-like molecules had shown higher survival rate in the presence of AI-2-like molecules (PCFS) than in ACFS or PB. Wild-type strains of *E. coli* O157: H7 can produce their own AI-2 molecules but still we observed the difference in the survival between ACFS and PCFS when used as a media.

SURVIVAL OF *luxS* MUTANT AND WILD TYPE *E. coli* O157: H7 STRAINS IN UNCOOKED GROUND BEEF EXTRACTS AT 4 °C IN THE PRESENCE OF PREFORMED CFS (PCFS), AUTOCLAVED CFS (ACFS), AND PHOSPHATE BUFFER (PB)

The survival of the *luxS* mutant (VS 94) and the wild type strain (ATCC 43895) at 4 ºC in ground beef extracts when amended with either PCFS, ACFS or phosphate buffer is shown in Figure 5. There were equal proportions of uncooked ground beef extracts and the amendments. The inactivation kinetics of the strains during storage at 4 ºC is shown in Table 3. The mutant strain showed over a 2.8 log reduction in the presence of PB as compared to a 2.2 log reduction observed in the wild type strain in PB. There was a significant difference ($P < 0.05$) in the survival of the *luxS* mutant strain (VS 94) in PB as compared to the wild type strain in the presence of PB. The *luxS* mutant showed approximately a 2.8 log reduction in PB as compared to 2.6 and 2.3 log unit reductions

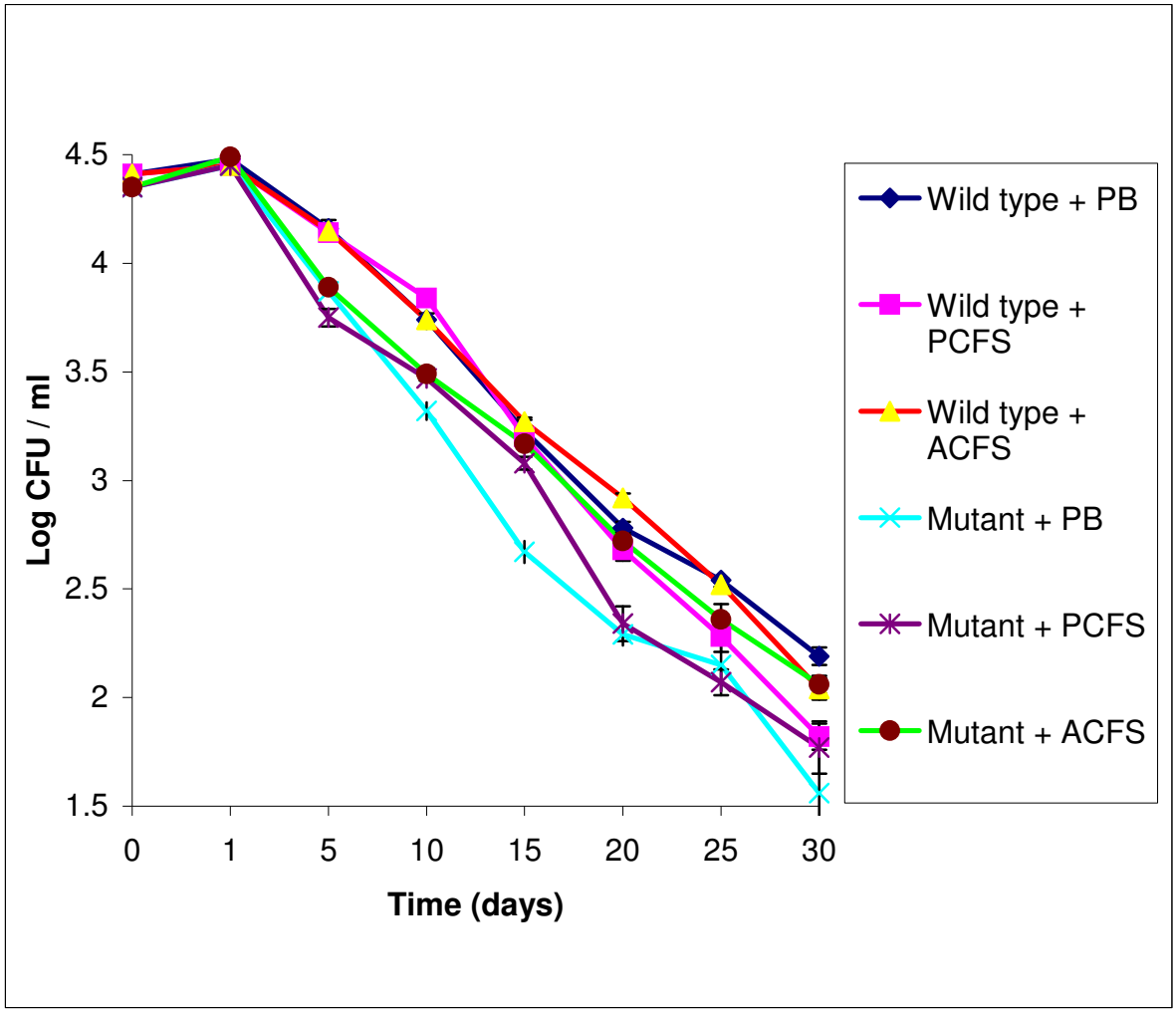

FIGURE 5. Survival of wild type and *luxS* mutant strain of *E. coli* O157:H7 for 30 days 4 ºC in the presence of uncooked ground beef extracts when amended with preformed CFS, autoclaved CFS, and phosphate buffer (PB).

TABLE 3 – INACTIVATION KINETICS OF *luxS* MUTANT (VS 94) AND WILD TYPE *E. coli* O157:H7 (ATCC 43895) AT 4°C IN THE PRESENCE OF UNCOOKED GROUND BEEF EXTRACTS WHEN AMENDED WITH PREFORMED CFS (PCFS), AUTOCLAVED CFS (ACFS), AND PHOSPHATE BUFFER (PB)

* Treatments[a]	[#] Regression equation[b]	r^2	[c]μ_d	[d]t_d	[e]k_d
W + UCGB + ACFS	y= -0.081+4.51	0.9953	-0.081	-3.72	-0.27
W + UCGB+ PCFS	y= -0.09+4.55	0.9877	-0.09	-3.34	-0.3
W + UCGB+ PB	y= -0.079+4.55	0.9902	-0.079	-3.81	-0.26
M+ UCGB+ ACFS	y= -0.08+4.37	0.985	-0.08	-3.76	-0.27
M + UCGB+ PCFS	y= -0.091+4.36	0.975	-0.09	-3.34	-0.3
M + UCGB+ PB	y= -0.095+4.35	0.9728	-0.095	-3.17	-0.32

*W = Wild type; M = Mutant type; PB= Phosphate buffer; ACFS= Autoclaved cell free supernatant in which AI-2 like activity was destroyed; PCFS= Preformed cell free supernatant having AI-2 like activity; UCGB= uncooked ground beef extracts;

[#] Mean of three line equation and kinetic parameters from the death periods (μ', t_d, k')

[+]r^2 = Pearson square coefficient; [c]μ_d = Slope from the equation, [d]t_d = death time; [e]k_d = death rate

in the presence of PCFS and ACFS respectively. Externally added AI-2-like molecules in the form of PCFS did not have any significant effect on the survival of wild type or mutant strain compared to heat degraded AI-2-like molecules (ACFS). No significant difference observed in the survival between PCFS and ACFS in the presence of uncooked ground beef extracts. Overall, both the wild type and *luxS* mutant strain of *E. coli* O157:H7 showed over a 2-log reduction in numbers over 30 days at 4 ºC in ground beef extracts. Based on the inactivation kinetic calculations there was no significant difference in the inactivation observed in either the mutant or the wild type strain when stored in ground beef at 4 ºC in the presence of PCFS, ACFS or phosphate buffer (Table 3). Also lower survival in the presence of uncooked ground beef extracts compared to one in pure media (PCFS, ACFS, P.B) indicates that *E. coli* O157: H7 cells can better survive in pure media condition than in the presence of uncooked ground beef extracts at refrigeration temperature.

SURVIVAL OF *luxS* MUTANT AND WILD TYPE *E. coli* O157: H7 STRAINS IN COOKED GROUND BEEF EXTRACTS AT 4 °C IN THE PRESENCE OF PREFORMED CFS (PCFS), AUTOCLAVED CFS (ACFS), AND PHOSPHATE BUFFER (PB)

The survival of the *luxS* mutant (VS 94) and the wild type strain (ATCC 43895) at 4 ºC in cooked ground beef extracts when amended with either PCFS, ACFS or PB is shown in Figure 6. The inactivation kinetics of the strains is shown in Table 4. The wild type *E. coli* O157:H7 strain, exhibited a 1.2 log reduction by the end of 30 days in the mixture of ACFS and cooked ground beef extract, while 1.8 log reduction was observed

in the mixture of PB and cooked ground beef extracts. The *luxS* mutant strain of *E. coli* O157:H7, was reduced by 1.4 log units by the end of 30 days in the presence of ACFS and cooked ground beef extracts, while there was over a 2.2 log reduction in the mutant strain in the presence of cooked ground beef extracts and PB. No significant difference was observed in the survival of wild type *E. coli* O157:H7 strain between PCFS and ACFS when mixed with cooked ground beef ($P > 0.05$). Likewise, no significant difference was observed in the survival of *luxS* mutant strain between PCFS and ACFS when mixed with cooked ground beef ($P > 0.05$). There was a significant difference in the survival of the *luxS* mutant strain (VS 94) in cooked ground beef extracts when amended with either PCFS or ACFS as compared to cooked ground beef amended with PB ($P < 0.05$). Higher survival was also observed in the wild type strain (ATCC 43895) in ground beef extracts amended with PCFS or ACFS as compared to cooked ground beef when amended with PB ($P < 0.05$), Table 4. Externally added AI-2-like molecules (PCFS) did not have a direct effect on the survival of wild type and mutant strain of *E coli* O157:H7 compared to heat-degraded AI-2-like molecules (ACFS). In the presence of cooked ground beef extracts, higher survival of wild type strain was observed

compared to *luxS* mutant strain for all conditions (PCFS/ACFS/PB) indicating a probable role of *luxS* gene in the survival of *E. coli* O157:H7 under cooked condition. In addition, higher survival was observed for wild type strain compared to mutant strain in the presence of cooked ground beef extracts mixed with PB was in concord with the results shown in Figures 4&5. Higher survival was observed in both wild type and *luxS* mutant strain of *E. coli* O157: H7 in the presence of cooked ground beef extracts when amended with PCFS, ACFS, or PB compared to uncooked ground beef extracts amended with PCFS, ACFS, or PB as shown in Figure 5, 4 and Table 3 &4 ($P < 0.05$). When compared with the study conducted using 100% of PCFS, as shown in Figure-4, lower survival rate ($\underline{P} < 0.05$) was observed in the presence of cooked ground beef extracts amended with PCFS for both wild type and mutant strains of *E. coli* O157: H7. In addition, when compared with the study conducted using 100% of ACFS and PB, no significant difference ($P > 0.05$) observed in the presence of cooked ground beef extracts when amended with ACFS or phosphate buffer.

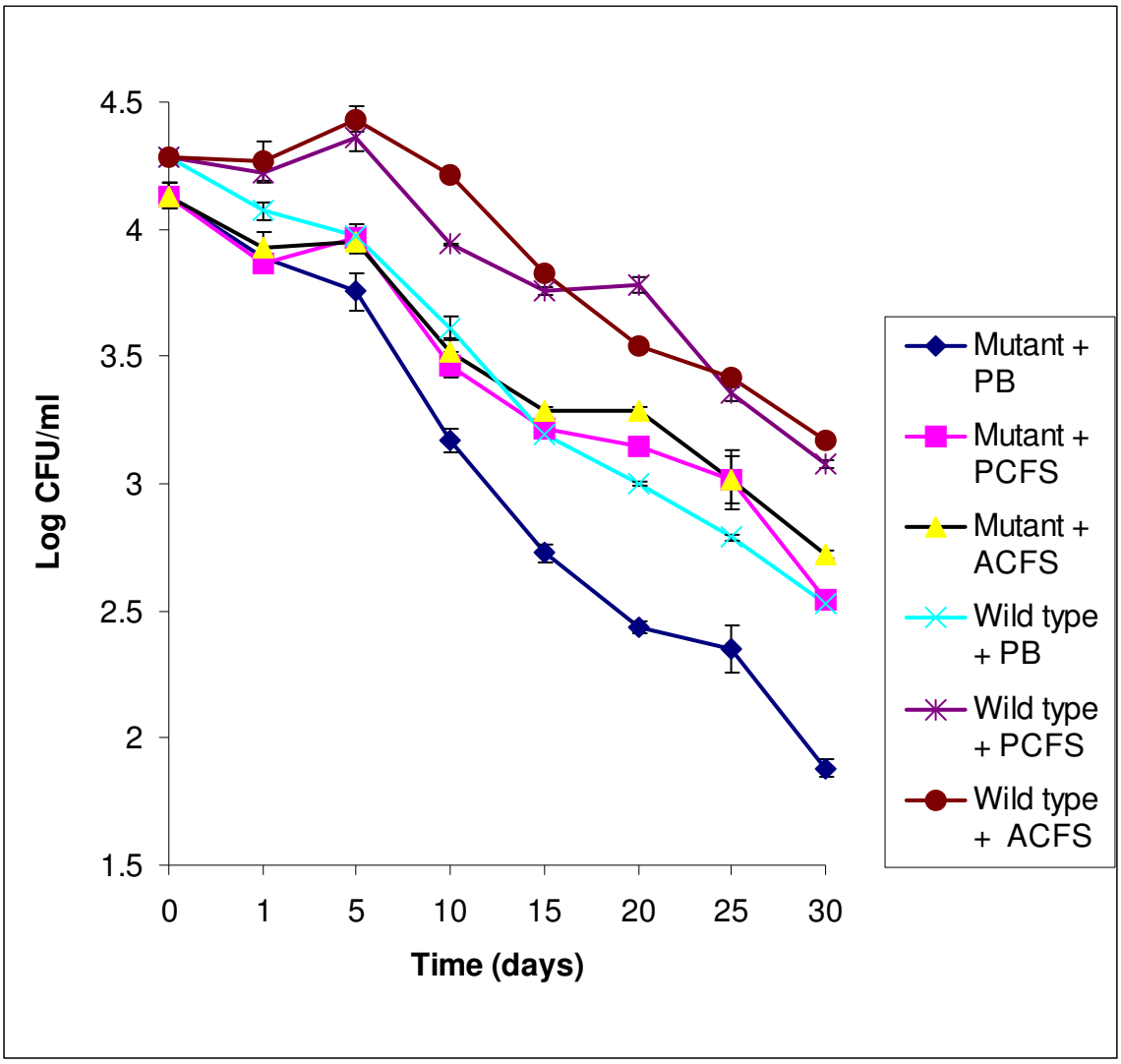

FIGURE 6. Survival of wild type and *luxS* mutant strain of *E. coli* O157:H7 for 30 days 4 ºC in the presence of cooked ground beef extracts when amended with preformed CFS, autoclaved CFS, and phosphate buffer (PB).

TABLE 4 – INACTIVATION KINETICS OF *luxS* MUTANT (VS 94) AND WILD TYPE *E. coli* O157:H7 (ATCC 43895) AT 4°C IN THE PRESENCE OF COOKED GROUND BEEF EXTRACTS WHEN AMENDED WITH PREFORMED CFS (PCFS), AUTOCLAVED CFS (ACFS), AND PHOSPHATE BUFFER (PB).

* Treatments[a]	[#] Regression equation[b]	r^2	[c]μ_d	[d]t_d	[e]k_d
W + CGB + ACFS	y= -0.041+4.43	0.9123	-0.041	-7.34	-0.14
W + CGB + PCFS	y= -0.05+4.44	0.8419	-0.05	-6.02	-0.17
W + CGB + PB	y= -0.058+4.2	0.9838	-0.058	-5.19	-0.19
M+ CGB + ACFS	y= -0.044+4.06	0.9449	-0.044	-6.84	-0.15
M + CGB + PCFS	y= -0.047+4.04	0.9371	-0.047	-6.4	-0.16
M + CGB + PB	y= -0.07+4	0.9678	-0.07	-4.3	-0.23

*W = Wild type; M = Mutant type; PB= Phosphate buffer; ACFS= Autoclaved cell free supernatant in which AI-2 like activity was destroyed; PCFS= Preformed cell free supernatant having AI-2 like activity; CGB= Cooked ground beef extracts

[#] Mean of three line equation and kinetic parameters from the death periods (μ', $t_{d,}$ k')

[+]r^2 = Pearson square coefficient; [c]μ_d = Slope from the equation,; [d]t_d = death time; [e]k_d = death rate

SURVIVAL OF *luxS* MUTANT AND WILD TYPE *E. coli* O157: H7 STRAINS IN AUTOCLAVED GROUND BEEF EXTRACTS AT 4 °C IN THE PRESENCE OF PREFORMED CFS (PCFS), AUTOCLAVED CFS (ACFS), AND PHOSPHATE BUFFER (PB)

Figure 7 represents the survival of the *E. coli* O157:H7 *luxS* mutant strain (VS 94) and the wild type strain (ATCC 43895) in autoclaved ground beef extracts at 4 ºC for 30 days when amended with PCFS, ACFS and PB. Table 5 represents the inactivation kinetics of these strains under these same conditions. The wild type *E. coli* O157: H7 showed a 2.6, 3.4, and 3.2 log reduction in the presence of ACFS, PCFS and PB respectively. The *luxS* mutant strain of *E. coli* O157:H7, VS 94 on the other hand showed a 3.6, 3.3, and a 4.3 log reduction under these same conditions. There were no significant differences in the survival of the mutant and wild type strains in autoclaved ground beef extracts under these experimental conditions ($P > 0.05$). Unlike results in Figures 4, 5, and 6, no significant difference ($P > 0.05$) was observed between the wild type and mutant strains of *E. coli* O157:H7 in the presence of autoclaved ground beef extracts mixed with PB. Interestingly, wild type of *E. coli* O157:H7 showed higher survival in ACFS compared to PCFS when mixed with autoclaved beef extracts.

In the presence of autoclaved ground beef extracts, a much higher ($P < 0.05$) death rate was observed compared to uncooked ground beef extracts, cooked ground beef extracts, and pure media (PCFS, ACFS or PB). For wild type and mutant strains of

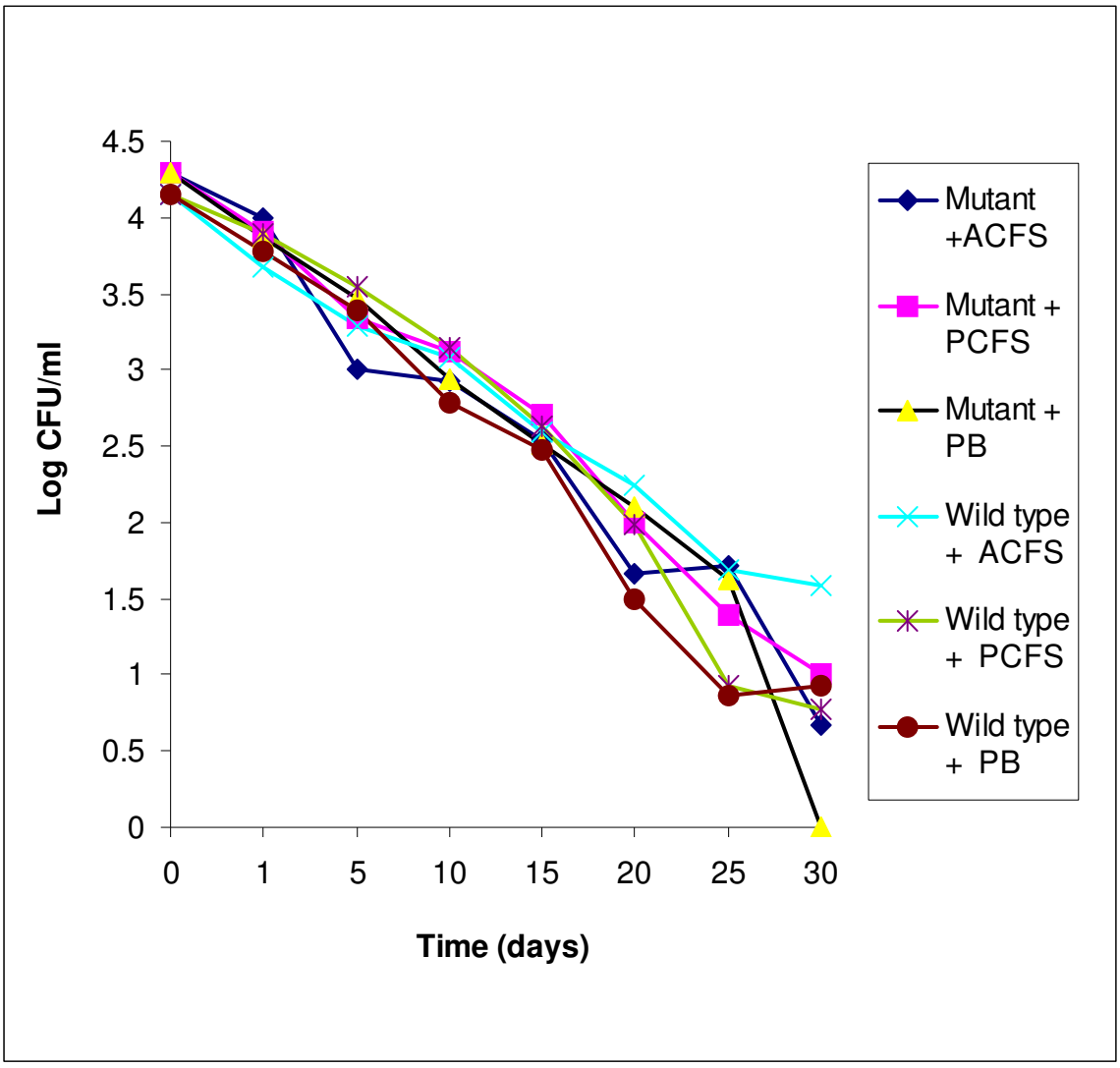

FIGURE 7. Survival of wild type and *luxS* mutant strain of *E. coli* O157:H7 for 30 days 4 ºC in the presence of uncooked ground beef extracts when amended with preformed CFS, autoclaved CFS, and phosphate buffer (PB).

TABLE 5 – INACTIVATION KINETICS OF *luxS* MUTANT (VS 94) AND WILD TYPE *E. coli* O157:H7 (ATCC 43895) AT 4°C IN THE PRESENCE OF AUTOCLAVED GROUND BEEF EXTRACTS WHEN AMENDED WITH PREFORMED CFS (PCFS), AUTOCLAVED CFS (ACFS), AND PHOSPHATE BUFFER (PB).

* Treatments[a]	[#] Regression equation[b]	r^2	[c]μ_d	[d]t_d	[e]k_d
W + AGB + ACFS	y= -0.081+3.89	0.9244	-0.081	-3.72	-0.27
W + AGB + PCFS	y= -0.111+4.13	0.9375	-0.111	-2.71	-0.37
W + AGB + PB	y= -0.113+3.98	0.9154	-0.113	-2.66	-0.38
M+ AGB + ACFS	y= -0.107+4.01	0.9375	-0.107	-2.81	-0.36
M + AGB + PCFS	y= -0.104+4.1	0.9478	-0.104	-2.89	-0.35
M + AGB + PB	y= -0.12+4.2	0.9442	-0.12	-2.51	-0.4

*W = Wild type; M = Mutant type; PB= Phosphate buffer; ACFS= Autoclaved cell free supernatant in which AI-2 like activity was destroyed; PCFS= Preformed cell free supernatant having AI-2 like activity; AGB= Autoclaved ground beef extracts;

[#] Mean of three line equation and kinetic parameters from the death periods (μ', t_d, k')

[+]r^2 = Pearson square coefficient; [c]μ_d = Slope from the equation, [d]t_d = death time; [e]k_d = death rate

E. coli O157:H7, a lower ($P < 0.05$) survival rate was observed in the presence of autoclaved ground beef extracts compared to uncooked ground beef extracts and cooked ground beef extracts when mixed with PCFS, ACFS, or PB. Also lower ($P < 0.05$) survival rate observed for both strain in the presence of autoclaved ground beef extracts when mixed with PCFS/ ACFS/PB compare to pure media (PCFS, ACFS, PB). The lower survival in the presence of autoclaved ground beef extracts was significant compared to all other conditions except autoclaved ground beef extracts mixed with ACFS and uncooked ground beef extracts mixed with ACFS did not show significant difference for wild type *E.coli* O157:H7.

INFLUENCE OF AI-2-LIKE ACTIVITY ON THE EXPRESSION OF *hha* AND *yadK* GENES IN WILD TYPE AND *luxS* MUTANT STRAINS OF *E. coli* O157:H7

The effect of AI-2-like molecules on the change in gene expression of *hha* and *yadK* gene was studied in context of meat-derived inhibitory compounds. The results are shown in Figures 8 through 14 (The changes in gene expression in the presence of AI-2-like molecules is represented in relation to that observed in LB broth, which was used as the baseline). In the *luxS* mutant strain, the presence of AI-2-like molecules in the form of PCFS results in a 1.9 and 2.8 fold increase in the expression for *haa* and *yadK* genes, respectively (Figure 8). This means that for each one-unit change in gene expression in the presence of LB broth, *haa* gene shows 1.9-fold change in gene expression while *yadK* gene shows 2.75-fold change in gene expression in the presence of AI-2-like molecules.

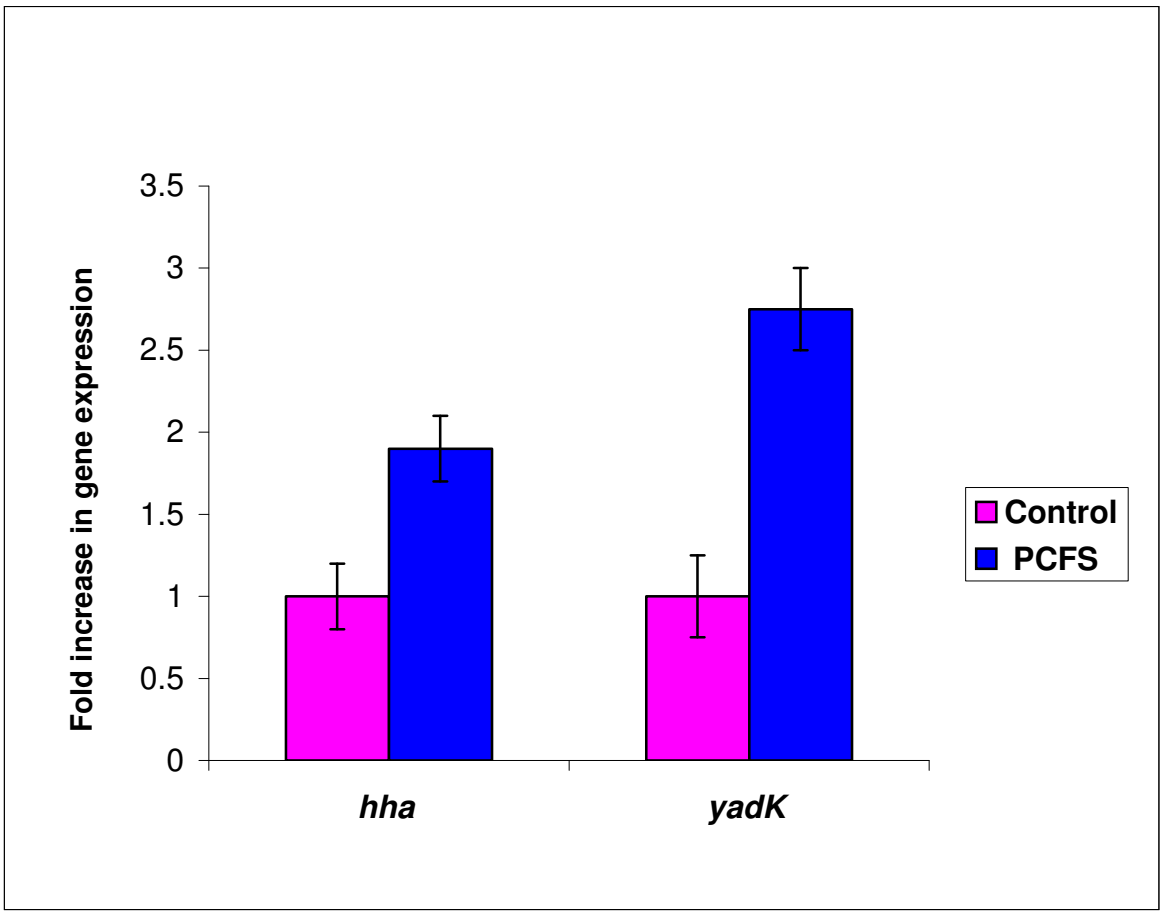

FIGURE 8. Induction of *hha* and *yadK* gene expression in *E. coli* O157:H7 *luxS* mutant strain in the presence of AI-2-like molecules added in the form of preformed cell free supernatant (PCFS).

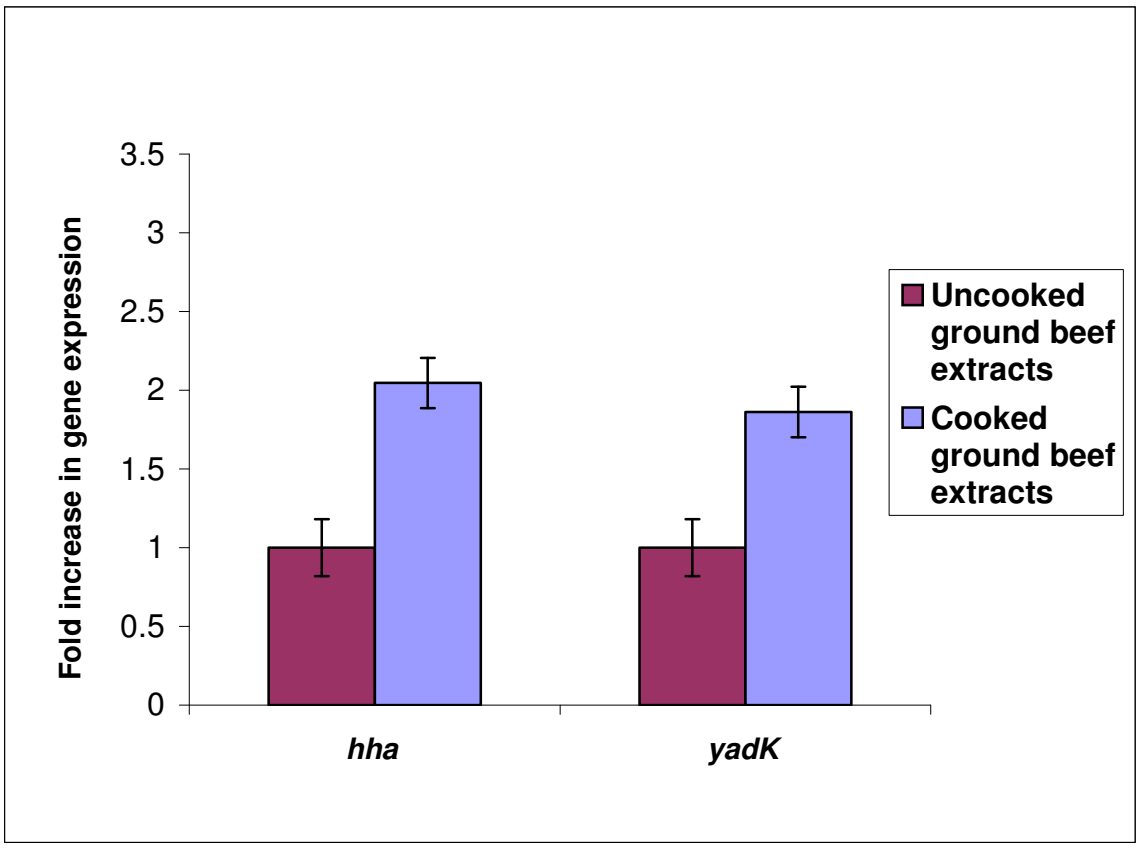

FIGURE 9. Induction of *hha* and *yadK* gene expression in *E. coli* O157:H7 *luxS* mutant strain of *E. coli* O157:H7 in the presence of uncooked and cooked ground beef extracts containing AI-2-like activity in the form of preformed cell free supernatant (PCFS).

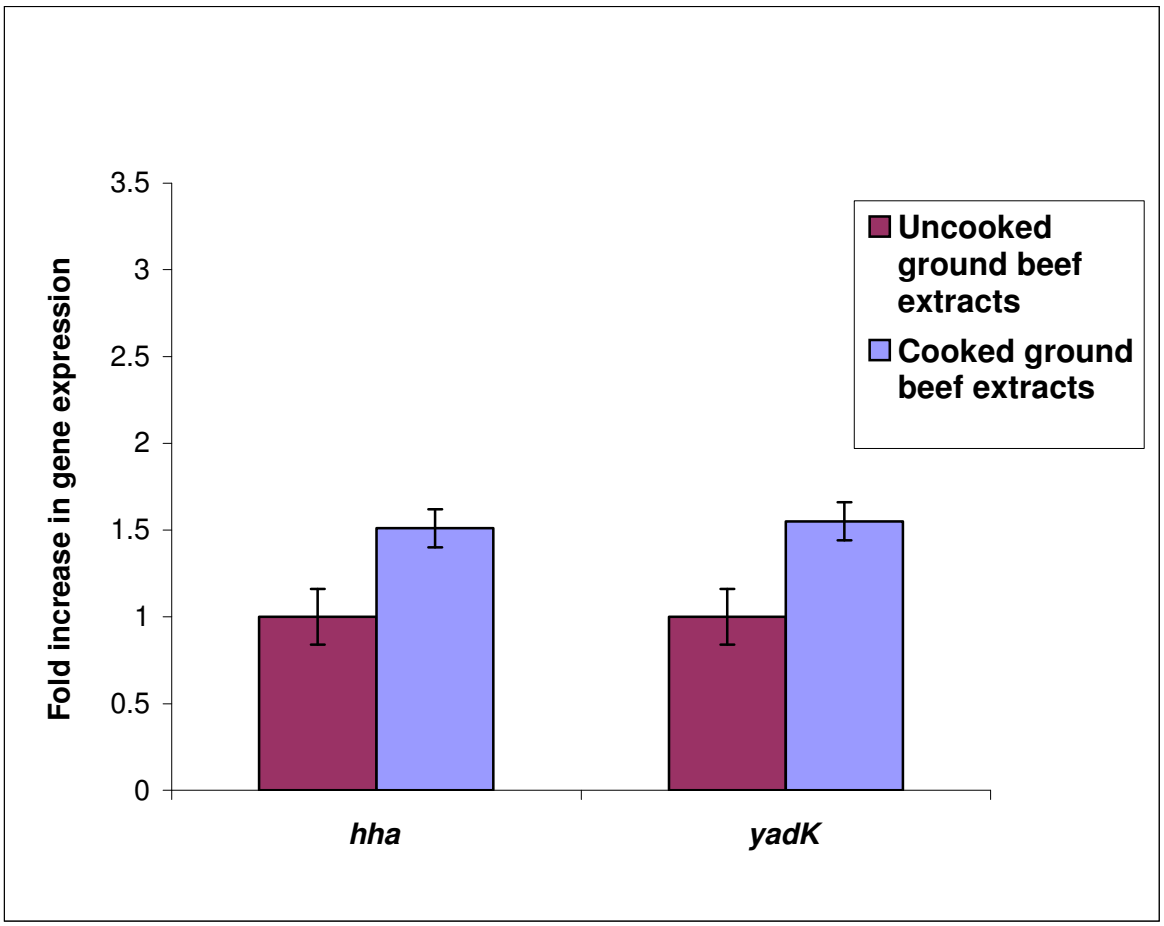

FIGURE 10. Induction of *hha* and *yadK* gene expression in *E. coli* O157:H7 *luxS* mutant strain in the presence of uncooked and cooked ground beef extracts when amended with Luria-Bertani (LB) broth.

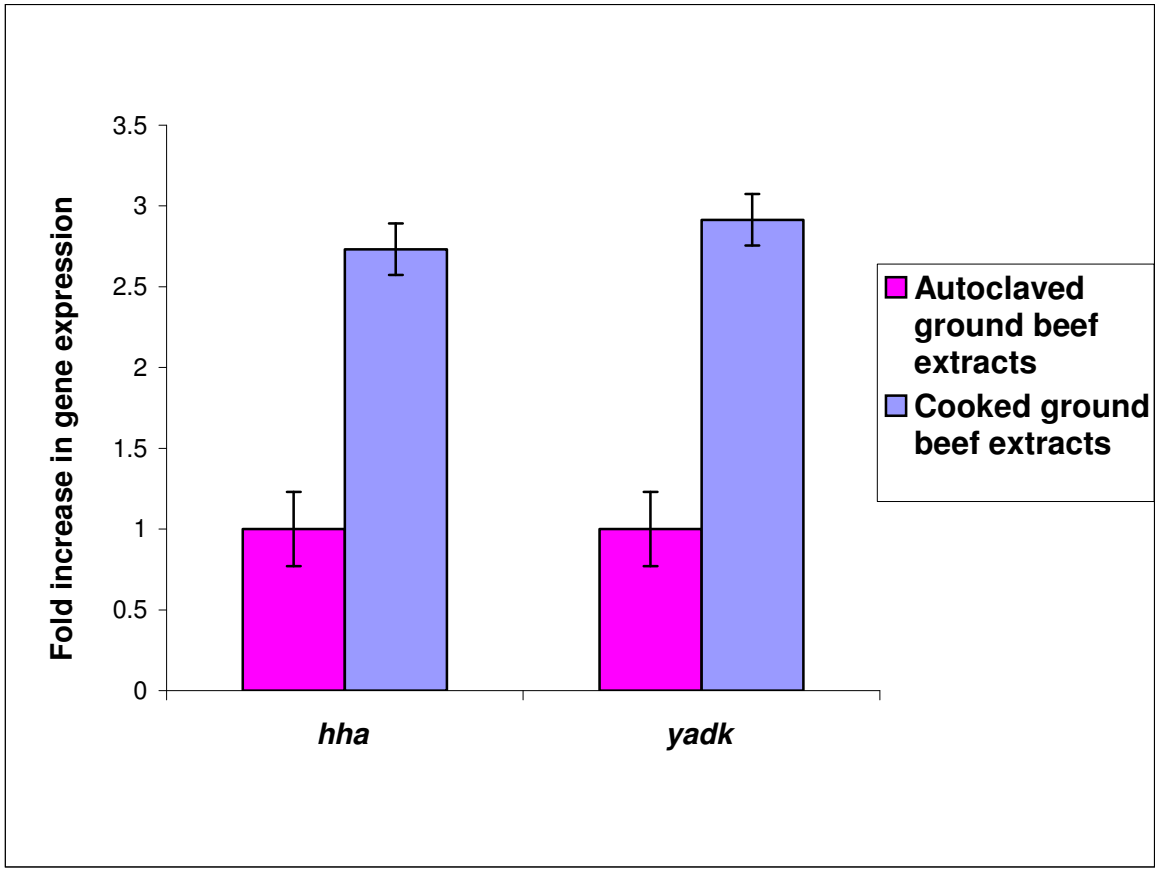

FIGURE 11. Induction of *hha* and *yadK* gene expression in *E. coli* O157:H7 *luxS* mutant strain of *E. coli* O157:H7 in the presence of autoclaved and cooked ground beef extracts containing AI-2-like activity in the form of preformed cell free supernatant (PCFS).

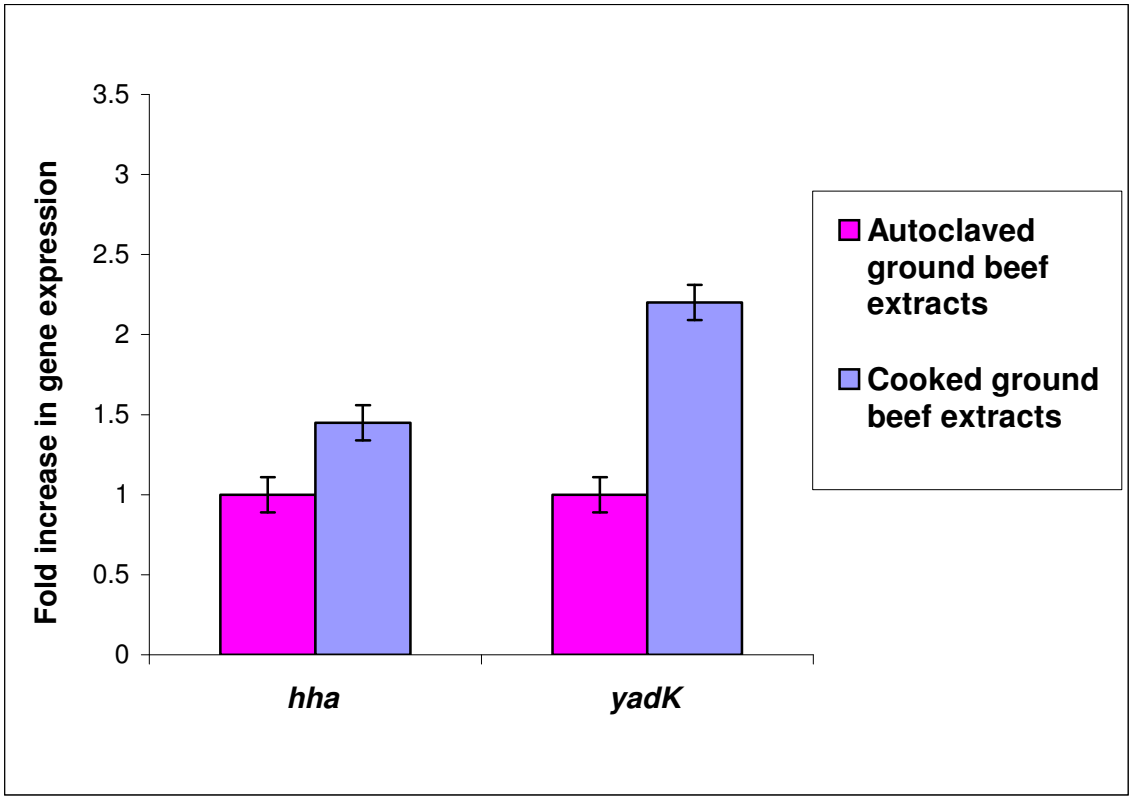

FIGURE 12. Induction of *hha* and *yadK* gene expression in *E. coli* O157:H7 *luxS* mutant strain in the presence of autoclaved and cooked ground beef extracts when amended with Luria-Bertani (LB) broth.

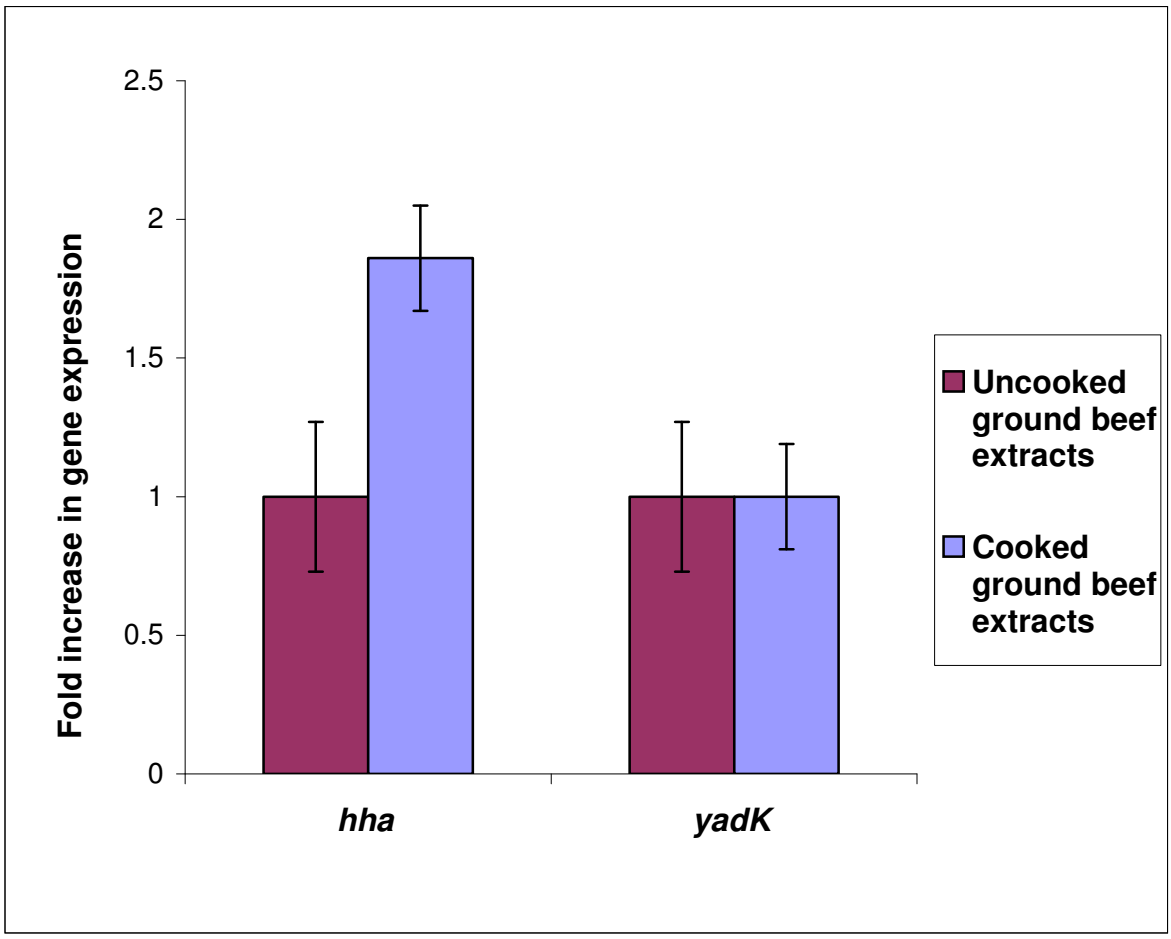

FIGURE 13. Induction *hha* and *yadK* gene expression in wild type strain of *E. coli* O157:H7 in the presence of AI-2-like activity in the form of PCFS in uncooked and cooked ground beef extracts.

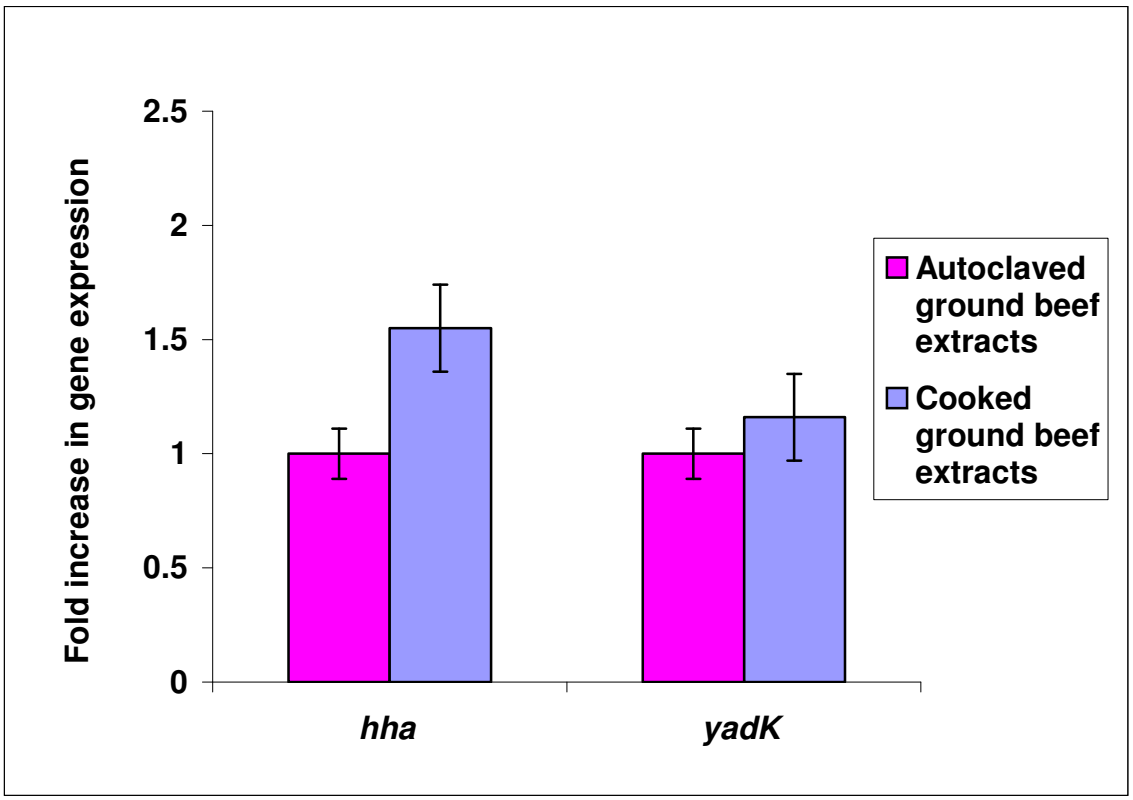

FIGURE 14. Induction *hha* and *yadK* gene expression in wild type strain of *E. coli* O157:H7 in the presence of AI-2-like activity in the form of PCFS in autoclaved and cooked ground beef extracts.

The use of anti quorum sensing compounds to control the quorum sensing mediated behavior of pathogenic organisms is one of the important emerging strategies in food and pharmaceutical industry. The ground beef contains inhibitory compounds that can interfere with cell-cell signaling mediated by AI-2-like molecules. Here we have reported the effects of inhibitory compounds present in the ground beef on the gene expression of *E. coli* O157: H7 for two selected genes. The *hha* and *yadK* genes that are upregulated in the presence of AI-2-like molecules were selected to see the effects of inhibitory compounds in context with AI-2-like molecules.

Fig 9 shows the increase in the gene expression of *hha* and *yadK* genes in the *luxS* mutant strain (VS 94) in cooked ground beef extracts containing AI-2-like activity supplied as PCFS as compared to the gene expression observed in uncooked ground beef extracts amended AI-2-like molecules. The *hha* gene shows about 2-fold increase in gene expression while *yadK* gene shows about 1.9-fold increase in gene expression in the presence of cooked ground beef extracts amended with PCFS as compared to 1.0-fold change in gene expression in uncooked ground beef extracts amended with PCFS.

The presence of Luria Bertani (LB) broth also enhances the expression of the *hha* and *yadK* genes in the *E. coli* O157:H7 *luxS* mutant strain (VS 94) (Figure 10). The presence of LB broth in the cooked ground beef conditions results in a 1.5-fold and 1.6-fold increase in the expression of the *hha* gene and *yadK* genes respectively in the cooked ground beef extracts as compared to 1-fold increase in the uncooked ground beef extracts. So both conditions, presence of AI-2-likes molecules (Fighre-9) or LB broth (Figure-10), shows higher gene expression in the cooked ground beef extracts compare

to uncooked ground beef extracts but the level of expression is different. When compared the data shown in Figure 9 and 10, the presence of AI-2-like molecules shows higher gene expression compare to LB broth for both selected genes in cooked condition over uncooked condition. In the presence of AI-2-like molecules, about 0.5 and 0.36 fold higher gene expressions were observed compared to LB broth for *hha* and *yadK* genes, respectively (comparison between Figure-9 and 10).

Figure 11 shows the difference in the gene expression of *hha* and *yadK* genes in the autoclaved and cooked ground beef extracts amended with PCFS. In the presence of PCFS (AI-2-like activity), the *hha* and *yadK* genes showed a 2.7 and 2.9-fold higher gene expression respectively in cooked ground beef extracts as compared to their 1.0-fold gene expression in autoclaved ground beef extracts.

Figure 12 shows the influence that LB broth has on the expression of the *hha* and *yadK* genes in the *luxS* mutant strain when exposed to autoclaved and cooked ground beef extract conditions. In the mixture of cooked ground beef extracts and LB broth, the *hha* and *yadK* genes in the *E. coli* O157:H7 *luxS* mutant strain exhibited a 1.5 and 2.2-fold increase in gene expression respectively compared to their 1.0- fold gene expression in the mixture of autoclaved ground beef extracts and LB broth.

Similar to results seen in the comparison between cooked and uncooked ground beef extracts, higher gene expression was observed in the presence of cooked over autoclaved ground beef extracts. The higher gene expression with the cooked extracts was seen in the presence of AI-2-like molecules and in the presence of LB broth. Compare to LB broth, the presence of AI-2-like molecules induced about 1.23 and 0.7

fold higher gene expression for *hha* and *yadK* genes respectively (comparison between Figure 11 and 12).

Figure 13 shows the changes in gene expression in the *hha* and *yadK* genes of the *E. coli* O157:H7 wild type strain (ATCC 43895) in cooked and uncooked ground beef extracts when amended with AI-2-like molecules in the form of a PCFS. The *hha* gene was expressed approximately 1.9 fold higher in cooked ground beef extracts amended with PCFS compared to uncooked extracts amended with PCFS. There was no change in the expression of the *yadK* genes in the cooked and uncooked ground beef extract conditions when amended with PCFS. Figure 14 shows the changes in gene expression of the *hha* and *yadK* genes in the *E. coli* O157:H7 wild type strain (ATCC 43895) in cooked and autoclaved ground beef extracts when amended with PCFS. The *hha* gene showed 1.6-fold higher gene expression in cooked ground beef extracts compared to 1.0-fold expression in autoclaved ground beef extracts when amended with PCFS. The *yadK* gene showed 1.2-fold higher gene expression in cooked ground beef extracts compared to 1.0-fold expression in autoclaved ground beef extracts when amended with PCFS. Consistent with the results obtained for mutant strain VS 94, wild type strain ATCC 43895 also showed higher gene expression with cooked extracts compared to uncooked and autoclaved, except tha *yadk* gene in cooked condition showed the same level of expression with uncooked ground beef extracts.

DISCUSSION

The presence of AI-2-like activity (as determined using the *V. harveyi* bioassay) in the wild type cells during the late log phase was evident in the wild type *E. coli* O157H7 strain ATCC 43895 (Figure 2). The *luxS* mutant strain (VS 94) exhibited extremely low levels of AI-2 activity (Figure 2). In wild type strain, the production of maximum AI-2-like activity was at mid exponential phase. As expected, a decrease in AI-2-like activity was observed in late exponential phase. A possible reason for decreased AI-2-like activity was the import and processing of AI-2 molecules by the Lsr transporter (Taga et al., 2003). Karina et al. (2005) and Taga et al. (2003) have mentioned that the degradation of AI-2-like activity in late exponential phase is due to the transport and processing of AI-2-like molecules within cells. It is hypothesized that that AI-2 autoinducer molecules are released in an early stage of growth and are metabolized in a later stage of growth (Winzer et al., 2002). The use of AI-2-like molecules in cell processes leads to the question of whether the AI-2 molecules are universal molecules or a part of metabolic activities.

There have been recent reports that certain foods possess AI-2-like activity (Lu et al., 2004; Smith et al., 2004). The AI-2-like activity present in foods is probably the result of microbial activity, although it is possible that the food products can contain compounds that can mimic the AI-2-like molecules. Efforts have been directed to find compounds that can mimic the AI-2-like molecules, but results are inconclusive (Lu et al., 2004; Teplitski et al; 2000; Teplitski et al., 2004). Previous work in our laboratory has showed that meat products, including ground beef patties, have some inhibitory

components that can interfere with the AI-2 signaling and that inhibitory compounds are heat labile (Lu et al., 2004; Jackob et al., unpublished data). We analyzed cooked and uncooked ground beef extracts for the presence of AI-2-like activity. However, levels of AI-2 like activity in meat products were negligible. There are two possibilities for the absence of AI-2-like activity in the cooked and uncooked ground beef extracts namely, AI-2-like molecules are not present in the beef extracts, or there are compounds in beef extracts that are interfere with the detection of the autoinducer molecules. To rule out the possibility that absence of AI-2-like activity in beef extracts is due to lack of AI-2 molecules, we performed the inhibition assay as mentioned by Lu et al. (2004). The presence of cooked and uncooked ground beef extracts appeared to have inhibited the response of the reporter strain (*V. harveyi*) to exogenous AI-2-like molecules. Thus, the possible reason for the absence of AI-2-like activity in beef extracts could be the inhibition of cell-cell signaling as mentioned by Lu et al. (2004). Furthermore, the removal of inhibitory compounds (as evidenced by the release of the inhibition) after autoclaving suggests that the inhibitory compounds are heat labile at autoclaving temperatures, but not at cooking temperature. In the presence of autoclaved ground beef extracts the *V. harveyi* reporter strain was able to sense exogenously provided AI-2-like molecules but not so in the presence of cooked ground beef extracts. We did not find the amount of PCFS needed to overcome the neutralizing effect of crude ground beef extracts (cooked and uncooked) on AI-2-like activity.

To determine the effects of AI-2-like activity (molecules) on the survival of *E. coli* O157:H7 in the context of inhibitory compounds, wild type and mutant strains were

exposed to cooked, uncooked, and autoclaved ground beef extracts in the presence of preformed cell-free supernatant (PCFS), autoclaved cell-free supernatant (ACFS) and phosphate buffer (PB). In general, the wild type strain showed higher survival than the mutant strain in PB. The higher survival of wild type cells compared to mutant in the PB suggests that the *luxS* gene (responsible for the production of AI-2 molecules) may play a key role in the survival of the bacterial pathogen. The *rpoS* gene is an alternative sigma factor and it is known to regulate various genes when the cells are under stress conditions (King et al., 2005; Gawande., 2005). Bacterial quorum sensing is thought to influence the *rpoS* gene regulation (Schuster et al., 2004; Hogardt et al., 2004). Up regulation in *rpoS* gene expression has been observed during quorum sensing cascade in *P. aeruginosa* (Schuster et al., 2004). However, there is still no precise picture available at the molecular level indicating how these quorum-sensing molecules are related to *rpoS* regulation (Schuster et al., 2004). *E. coli* O157:H7 cells exposed to PB are presumable under starved condition (due to the lack of nutrients in the buffer). The higher survival of the wild type cells compared to mutant strain suggests that the presence of *luxS* gene might be facilitating the induction of *rpoS* gene in stress condition. The *luxS* mutant strain, VS94, cannot produce AI-2-like molecules but can sense it (Sperandio et al., 2001). We hypothesized that if AI-2-like molecules are involved in the survival of *E. coli* O157:H7, then the presence of PCFS containing AI-2-like molecules should result in higher survival. As expected, higher survival was observed in the presence of 100% PCFS compared to 100% of ACFS, indicating that AI-2-like molecules may play a key role in survival (Figure 4). Higher survival in the presence of

PCFS is likely the result of the induction of stress related genes in the presence of AI-2-like molecules. Wild type strain ATCC 43889 can produce and sense their own AI-2-like molecules. We observed higher survival in the presence of externally added AI-2-like molecules in the form of PCFS compared to ACFS. This is probably because ACFS has enough nutrients in the preparation for the bacterial cells and thereby preventing the cells from going into a starvation condition. This can ultimately does not result in either intra or neither extra cellular production of AI-2-like molecules nor the induction of any survival related genes. Also the externally added AI-2-like molecules in the form of PCFS were produced under optimal conditions (37°C). We should not expect the production of same level of AI-2-like activity in the ACFS at refrigeration temperature (4°C) as seen in PCFS under optimum conditions. The lower survival for wild type strain in the presence of ACFS compared to PCFS is probably because of the less AI-2-like molecules in ACFS compared to PCFS.

No significant difference was observed in the survival of cells treated with PCFS and ACFS in the presence of uncooked ground beef extracts (Figure 5). These can be explained by the ability of uncooked ground beef extracts to inhibit cell-cell signaling mediated by AI-2-like molecules. It is also likely that the presence or absence of AI-2-like molecule did not make a difference in cell survival, as the cells could not use the AI-2-like molecules to mediate a stress response in the presence of inhibitors. If the *luxS* gene is involved in the survival, then we should expect higher survival in the wild type strain compared to mutant. We did not find much difference in the survival between the wild type and mutant strains under the different conditions (Figure 5). The inhibition of

cell-cell signaling might be preventing the *luxS* gene from regulating other survival related genes. These results suggest that the interference of cell-cell signaling results in the lower survival of the bacteria in the presence of uncooked ground beef extracts.

In the presence of cooked ground beef extracts, we did not find a significant difference in survival of cells treated with PCFS and ACFS (Figure 6). The cooking of the beef extract did not completely destroy the inhibitory compounds. The higher survival of the wild type strain compared to mutant is very evident. These results suggest that in the presence of cooked ground beef extracts, stimulation of the *luxS* gene favors the survival of wild type strain. Media composition is known to affect the survival of *E. coli* O157:H7 (Mamani et al., 2003). Under the same media condition (presence of cooked ground beef extracts) the wild type strain showed higher survival compared to the mutant strain that strongly suggests that the *luxS* gene is probably playing a key role in survival. Also, the higher survival observed for wild type strain compared to mutant in the presence of cooked ground beef extracts mixed with phosphate buffer indicates that *luxS* gene is favoring the survival of *E. coli* O157:H7 in cooked ground beef extracts (Figure 6). The over-all increase in the survival rate in cooked ground beef extracts compared to uncooked ground beef extracts may be because of the some degradation of inhibitory compounds at cooking temperature. The degradation of inhibitory compounds might have favored cell-cell communication and, ultimately, the higher survival rate. Higher survival in the presence of cooked ground beef extracts compared to uncooked ground beef extracts indicates that the cooking condition by itself can favor the higher survival of *E. coli* O157: H7.

In the presence of autoclaved ground beef extracts we observed less *E.coli* O157:H7 survival as compared to its survival in the presence of cooked and uncooked ground beef extracts. Autoclaving destroys the inhibitory compounds present in the uncooked ground beef extracts as shown in Figure 3. If autoclaved ground beef extract favored cell-cell signaling, there should be a higher survival rate in the presence of AI-2-like molecules (PCFS). Furthermore, as observed in the case of cooked ground beef extracts, a greater survival of the wild type cells compared to mutant should have occurred in the presence of autoclaved ground beef extracts. We, however, did not find higher survival for wild type cells compared to mutant in autoclaved beef extracts (Figure 7). Autoclaving can be considered to be a very harsh time-temperature treatment. It is possible that the autoclaved beef extract would have a completely different physical and chemical composition as compared to uncooked and cooked ground beef. Mamani et al. (2003) have reported that the survival of *E. coli* O157:H7 at 4°C also depends on the media used. It may be possible that the components created in autoclaved ground beef extracts do not favor the survival of *E. coli* O157:H7. The effect of these autoclave-generated components may have been the reason why there was a lower *E. coli* O157:H7 survival in the presence of autoclaved ground beef extracts.

AI-2-like molecules are known to be involved in the regulation of gene expression in many bacterial species. In *Escherichia coli,* about 6-10% of total genes are known to be up or down regulated ((Delisa et al., 2001; Sperandio et al., 2001). These investigators have shown that multiple genes can be differentially expressed when an AI-2 deficient (*luxS* mutant) strain is complemented by an exogenous source of AI-2-

like molecules. In *E. coli* O157:H7, the *hha* gene is involved in the production of hemolysin expression modulating protein, while *yad*K gene is involved in the production of putative fimbrial-like protein (Jubete at al., 1995; Goebal et al., 1982; Brauner et al., 1995; Welch et al., 2002). Furthermore, Delisa et al. (2001) have reported a 11.1 and a 3.8 fold up regulation of the *hha* and *yadK* genes, respectively, in the presence of AI-2-like molecules. We selected *hha* and *yadK* genes to study the effect of AI-2-like molecules on gene expression. When an AI-2-deficent strain was complemented with exogenous AI-2-like molecules, 1.9 and 2.75-fold increases in gene expression were observed for, *hha* and *yadK* respectively (Figure 8). There was generally less increase in gene expression in the presence of AI-2-like molecules for these genes compared to results obtained by Delisa et al (2001). Even though the magnitude of change was different our results are consistent with the previously published results (Delisa et al., 2001). Both *hha* and *yadK* genes were up regulated in the presence of AI-2-like molecules (Figure 8). The differences observed in the level of induction of the selected genes between our results and what was published earlier could be attributed to a number of methodological differences between the experiments conducted by Delisa et al (2001) and the present study. Delisa et al. (2001) used microarray analysis for gene expression, while we employed a more specific and accurate real-time PCR approach. The results obtained by real-time PCR are generally more reliable than microarray analysis. Moreover, Delisa et al. (2001) used the *E. coli* MDAI2 strain, while we used the *E. coli* O157:H7 VS 94 strain. Delisa et al. (2001) used an incubation temperature of 30°C with 20 min exposure to PCFS, while we used 37°C incubation with a 25-min

exposure time. The conditioned media (PCFS) used by Delisa et al. (2001) was prepared from supernatant collected from cells at OD_{600nm} of 3.0, while the OD_{600nm} of cells that was used in this study was 1.0. The use of culture supernatant obtained during stationary or late logarithmic phase culture to treat cells of logarithmic phase can lead to big differences in the results (Ren et al., 2004). To avoid such errors we used the same OD_{600nm} (i.e., 1.0) to both harvest the cells and obtain the supernatant for the gene expression studies.

Most of the human illnesses associated with pathogenic bacteria are because of the consumption of microbiologically unsafe foods and water. Over the last decade, many bacterial processes have been shown to be controlled by quorum sensing molecules and much work has been done on the molecular level (Smith et al., 2004; Miller et al., 2001). However, at the same time, the study of these quorum-sensing molecules in food systems has almost been neglected. So, rather than just looking at the effect of AI-2-like molecules on bacterial gene expression, we decided to study the influence of AI-2-like molecules on bacterial gene expression as influenced by a food system. We studied the effect of AI-2-like molecules in different types of ground beef extracts in context with inhibitory compounds contained in ground beef. We observed higher gene expression for both genes in the presence of cooked ground beef extracts compared to uncooked and autoclaved ground beef extracts. The higher gene expression in cooked ground beef extracts was consistent with results obtained in the survival study. We concluded that the presence of inhibitory compounds in the uncooked ground beef extracts represses the expression of both genes, while the some inactivation of inhibitory

compounds in cooked ground beef extracts allows for expression of both genes. Also, if autoclaving inactivates inhibitory compounds in the ground beef extracts, there should have been higher gene expression for both genes. However, this was not the case. Similar to the results obtained during the survival studies, cooking of the ground beef extract resulted in higher gene expression for the 2 selected genes compared to both uncooked and autoclaved extracts. During the preparation of autoclaved ground beef extracts it is possible that protein denaturation and a wide variety of chemical and physical changes take place, which could have completely different consequences as compared to uncooked and cooked ground beef extracts. The lower gene expression observed in autoclaved ground beef extracts as compared to cooked ground beef extracts (Figure 11,12, and 14) suggest the probable influence that the suspending medium has on gene expression similar to what was observed during the survival studies.

In general, in the presence of Luria Broth (LB) broth cooked ground beef extracts induced higher gene expression for both of the selected genes compared to the effects of uncooked and autoclaved ground beef extracts (Figure 10&12). Thus it arises the question, “What is the role of AI-2-like molecules, if in the presence of LB broth by itself, the cooked ground beef extracts shows higher gene expression?” To answer this question we compared the gene expression in cooked ground beef extracts amended with PCFS and LB broth. When compared with the LB broth, the presence of AI-2-like molecules induced higher gene expression in cooked ground beef extracts over uncooked and autoclaved ground beef extracts (Figure 9-14). Thus it can be concluded that higher gene expression in cooked ground beef extracts is not only because of cooking condition

but also the combine effect of AI-2-like molecules. Wild type strain ATCC 43895 produce highest AI-2-like activity when its OD_{600nm} is in between 1.0-1.2 and in our experimental design we gave different treatments (exposure to AI-2-like molecules or LB broth) at OD_{600nm} of 1.0. As wild type cells can produce their own maximum AI-2-like molecules at 1.0 OD, we only treated our samples with AI-2-like molecules and not with LB broth. The up-regulation of *hha* from a pathogenicity standpoint suggests that it can regulate the expression of LEE region and *hilA*. While the up-regulation of *yadK* gene suggest that it increase the function related to motility and secretion system. Microarray based analysis is very critical to come up with a precise picture that how the cooking conditions can regulates the gene expression. The increase in the gene expression in the presence of AI-2-like molecules indicates that the AI-2-like molecule could play vital role in regulating gene expression in cooked ground beef extracts condition.

CONCLUSION

Cooking has always been considered as safe practice, because of its ability to destroy harmful pathogens such as *E. coli* O157:H7. However, these results suggests that if there is an initial heavy pathogen load coupled with improper cooking, it is possible that these cells could exhibit extended survival and higher gene expression of pathogenicity related genes. An initial high load of such pathogen, improper cooking, and post cooking contamination are some of the conditions likely to promote the presence of *E. coli* O157:H7 in cooked ground beef products. Based on these results it appears that the survival and expression of selected genes (*hha* and *yadK*) are higher in cooked conditions when compared with uncooked and autoclaved condition. The results also suggest that AI-2-like molecules could influence bacterial survival of *E. coli* O157:H7 in food systems. The cell-signaling inhibitory compounds present in uncooked ground beef extracts may have a potential to be used as a novel pathogen intervention strategy to control *E. coli* O157:H7 and other food-borne pathogens in food products. Further studies should be conducted to completely understand the relationships between AI-2 like activity, pathogen survival and expression of key virulence related genes. These interactions need to clearly delineated to effectively limit the proliferation and toxicity of enteric bacteria in meats and other foods.

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WORK EXPERIENCE

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